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# A Pharmacologic Investigation Of The Antiinflammatory Activity Of Cryogenine

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# A PHARMACOLOGIC INVESTIGATION OF THE ANTIINFLAMMATORY ACTIVITY OF CRYOGENINE

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Abstract of Dissertation

This study was conducted in an attempt to define the antiinflammatory efficacy of cryogenine, the major alkaloid of Heimia salicifolia Link and Otto. The antiinflammatory effects produced by cryogenine, phenylbutazone, indomethacin, mefenamic acid, paramethasone, hydrocortisone and 6-mercaptopurine were compared considering the sequential changes in various motameters during the course of adjuvant-induced polyarthritis in rats. Polyarthritis was induced by the subplantar injection of a suspension of heat-killed Mycobacterium butyricum in mineral oil into the right hind paw of Sprague-Dawley rats. The manifestations of the disease were monitored in terms of hind paw swelling, subjective appearance of secondary lesions, impairment of body growth, and various hematological/serological changes. Effects produced by cryogenine in ganglia and acute cardiovascular interactions with various mediators of inflammation and vasoactive agents were also investigated in the rat. The efficacy of cryogenine in delaying ultraviolet-induced erythema and drug-induced bronchoconstriction in the guinea pig was determined. Results showed cryogenine to be effective in suppressing the development of adjuvant-induced polyarthritis in doses which appeared to be devoid of any toxic manifestations. Cryogenine was effective in permitting a partial restoration of the normal rate of body weight gain. The therapeutic effects of cryogenine in adjuvant-injected animals were reflected in almost normal lymphocyte/neutrophil ratios in drug-treated animals. Moreover, cryogenine suppressed adrenocortical overactivity as evidenced by near normal thymus and adrenal weights. Cryogenine markedly inhibited increases in sedimentation rate and decreases in serum turbidity. These studies have shown cryogenine to possess significant antierythemic activity, but to be ineffective in inhibiting bradykinin-, histamine-, and serotonin-induced brocho-constriction in the guinea pig. Cryogenine was essentially devoid of any ganglionic activity and produced no effect on resting blood pressure or on cardiovascular responses to epinephrine, acetylcholine, histamine, or bradykinin. Although cryogenine is structurally unrelated to any known class of antiinflammatory, antipyretic or analgesis compounds, it has been shown to be effective in several experimental inflammatory conditions which respond to the antiinflammatory drugs presently in clinical use. Its nonspecificity of action against neuro- and tissue hormones suggest that its antiinflammatory activity is not related primarily to inhibition of acute phase reactants or to peripheral neurotropic effects. Involvement of the pituitary-adrenal axis in the therapeutic efficacy of cryogenine seems improbable.

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## INTRODUCTION

*Heimia salicifolia* Link and Otto (family Lythraceae) is a relatively small shrub, 0.5 to 3 m in height, growing generally in moist areas and indigenous to a widely dispersed area extending from Mexico to the borders of Argentina, including Uruguay, Paraguay and southern Brazil (1). Some confusion has existed as to the taxonomic status of the genus *Heimia* because of variability in the alkaloidal content of the plant. Several investigators accept the existence of two distinct species of *Heimia*: *H. salicifolia* found in the northern range (Mexico) and *H. myrtifolia* occurring as the southern species (Argentina) (2). This distinction, however, is not universally accepted. Moreover, a recent comparison of the alkaloid spectra in the two species has shown them to be essentially identical; this is in accord with the suggestion by other authorities that *Heimia* is actually a monotypic genus. The variability in alkaloid distribution in the two species has been ascribed to climatic or other environmental variations.

The plant is known under numerous common names including "quiebra arado" (plow-breaker) in Argentina, "abre-o-sol" (sun-opener) in the Rio Grande del Sur, "herva de la vida" (herb of life) in Brazil and "sinicuichi" (twisted foot) in Mexico.

The folklore associated with the use of the plant is of special interest. In Mexico "sinicuichi" is applied both to the plant and to a fermented decoction produced from its leaves. Blomster et al. (3) and Tyler (4) have presented a summation of numerous citations describing the use and interesting effects attributed to "sinicuiche", the "magic drink causing oblivion." "The drink is prepared by macerating slightly wilted leaves in water, after which the juice is strongly expressed, allowed to ferment, and clarified in the sun" (4). "Imbibition of the beverage produces a feeling of giddiness, a mild stupefaction, lassitude and a sensation whereby the surroundings become darker, and the world becomes infinitely smaller. Voices of those nearby, the ringing of bells, and other sounds seem to come from great distances. The drinker seems to have a distorted sense of time and place and is forgetful. It is stated that he often retells of earlier events as if they had just taken place. The whole state is characterized as one of pleasant drowsiness. No 'hangover' or other detrimental effect is noted the next day. The single abnormal symptom accompanying overindulgence is a peculiar

yellow vision. Although habitual excessive use of 'sinicuiche' is apparently detrimental to the memory, its users are said to be able to remember, with great exactitude, happenings and experiences of their remote past. In some cases, events which took place before the birth of the individual, but experienced by his ancestors, are supposedly recalled" (3).

In 1958, Hegnauer and Herfst (5) confirmed the occurrence of appreciable amounts of alkaloids in the leaves of Heimia salicifolia. In 1963, Blomster et al. (3) reported the isolation of five alkaloids from the plant, which were designated lythrine, sinicuichine, heimine, sinine, and cryogenine[1]. The following year two additional alkaloids, nesodine and lyfoline, were isolated and purified from H. salicifolia by Appel et al. (6).

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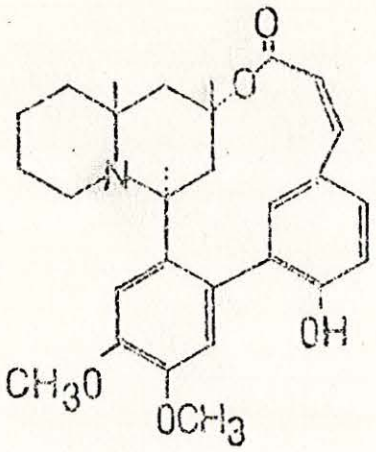
[1] Chemical studies conducted by Ferris et al. (21) concerning the Lythraceae plant family described the isolation of certain alkaloids from Decodon verticillatus. After the isolation and purification of the alkaloids of Heimia salicifolia had been reported, similarities between these bases and those isolated from Decodon were noted (3). Blomster et al. established the empirical formula for cryogenine and pointed out its near equivalency with vertine, isolated from Decodon verticillatus by Ferris. Subsequently, Zacharias et al. (22) indicated that vertine was identical with cryogenine. The chemical structure for cryogenine (vertine) has since been proposed (23) and confirmed (24). Furthermore, it should be noted that cryogenine (MW = 435.53), the major alkaloid isolated from Heimia salicifolia Link and Otto, is not the registered trade name product Cryogénine (phenylsemicarbazide, (MW = 151.2) a specialty of Lumière of Lyons, France, and distributed by Laboratoires Sarbach of Châtillon, France.

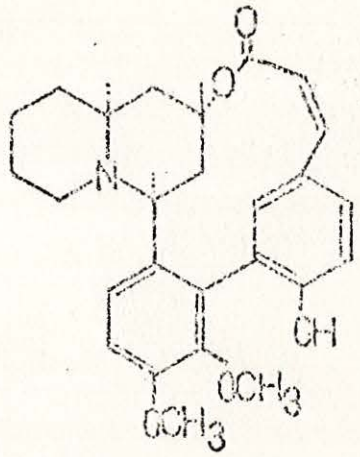
During a recent isolation of cryogenine for pharmacologic evalutation, an eighth alkaloid was isolated from the plant and named heimidine (7). The known structures of four of the alkaloids are illustrated in Figure 1. Based on current extraction techniques, cryogenine content in the dried overground plant approximates 0.035% (8).

The uses of the plant in folk medicine are numerous and diverse. In Brazil, according to Penna (9), the plant finds use as an antisyphilitic, an insecticide, a general depurative and as a sudorific. Calderon (10) notes that the plant has been used as a cure for dysentery and indigestion, in the treatment of bronchitis and other chest ailments and as an appetite stimulant. The plant is also used by women for inflammation of the womb and after delivery to "close the womb." In Mexico, Heimia salicifolia has found widespread use in local medicine because of its reputed hemostatic, diaphoretic, diuretic, purgative and astringent properties. In addition, an infusion of the plant has found utility in facilitating the healing of ulcers and as an antiinflammatory in Rhus dermatitis (11).

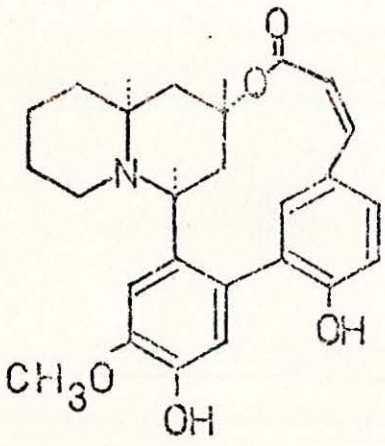
In 1960, results of a preliminary pharmacologic evaluation of extracts containing the total alkaloids of Heimia salicifolia and of purified cryogenine, itself, indicated that cryogenine mimicked qualitatively and quantitatively the actions of the total alkaloidal fraction of the plant (12). In both unanesthetized rats and dogs, cryogenine produced a qualitatively unique central nervous system depressant action characterized by a reduction in



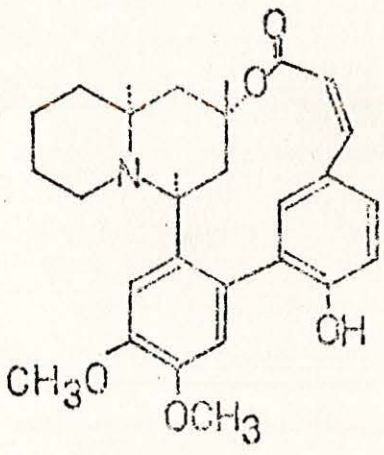

CRYOGENINE




NESODINE




LYFOLINE




LYTHRINE

Figure I.-- Structurally defined alkaloids of Heimia Salicifolia

motor activity, general passivity and mild hypothermia. Coupled with these "tranquilizing" properties there was little evidence of ataxia, muscle dyscoordination, analgesia, blepharoptosis and muscle relaxation. Neither convulsions nor parkinson-like tremors were noted at lethal dose levels. In addition, no apparent alterations in resting blood pressure were observed in anesthetized dogs and cats.

On isolated smooth and skeletal muscle preparations, cryogenine acted as an antispasmodic blocking acetylcholine-induced contractions in both frog rectus and ileum preparations (guinea pig, rabbit). Subsequent kinetic studies indicated the alkaloid to be a noncompetitive inhibitor of acetylcholine (13). Attempts to protect against both electrically- or pentylenetetrazol-induced convulsive seizures were not successful, although cryogenine appeared to significantly delay the onset of death in pentylenetetrazol-injected rats. The results of this study also showed that cryogenine would block nervous conduction in isolated frog sciatic nerve only at relatively high molar concentrations.

To explore and corroborate the psychotropic potentialities suggested by the bizarre folklore, rats were subjected to various models of conditioned avoidance-escape behavior (13). Cryogenine at relatively low, nonataxic dose levels was shown to selectively suppress "anxiety" in both discrete and continuous discriminative and nondiscriminative

situations. A consideration of these results together with the lack of serious side effects (hypotension, skeletal muscle involvement, extrapyramidal effects) produced by the alkaloid indicated cryogenine to be qualitatively unique relative to the present clinically employed tranquilizers (when such compounds are administered at comparable anxiety-reducing dosages).

When sufficient quantities of the other pure alkaloids from Heimia salicifolia became available Kaplan and Malone (14) initiated a comparative pharmacologic study of these alkaloids with cryogenine. Gross observational screening in unanesthetized rats confirmed earlier studies of the qualitative symptomatology produced by cryogenine. Lythrine hydrochloride, heimine and lyfoline bases were found to be inactive except at lethal dosages. Nesodine appeared to possess some cryogenine-like sedative activity, but only at relatively high dose levels. In cardiovascular studies in intact anesthetized dogs, both cryogenine and nesodine appeared to inhibit the pressor effects produced by exogenously administered epinephrine; however, kinetic studies utilizing rat vas deferens demonstrated an apparent lack of specificity for adrenergic receptors by both alkaloids. One interesting observation was the facilitation by cryogenine of smooth muscle contractions produced by serotonin on isolated rat vas deferens. Additional isolated smooth muscle studies demonstrated the noncompetitive antihistaminic, antimuscarinic, and anticholinergic

properties of cryogenine. In addition, cryogenine appeared to possess little or no cholinesterase inhibitory activity (15).

In 1966, in a comprehensive evaluation of the pharmacologic actions of numerous plants used as local medicines in Mexico, Jiu (16) confirmed the central nervous system depressant properties produced by ethanol extracts of H. salicifolia. He further noted a significant anti-inflammatory action produced by extractives of the plant in two separate models of inflammation (yeast-induced foot edema in the rat, and cotton pellet granuloma in the guinea pig. This confirmation that cryogenine might be a new chemical prototype for antiinflammatory activity intensified ongoing experiments at The University of Connecticut by Kaplan and co-workers, and these studies were published in 1967 (15). Cryogenine was found to be partially effective in limiting the inflammatory responses induced by plantar injection of mycobacterial adjuvant and was found to have significant antiphlogistic activity against carrageenin-induced pedal edema. Cryogenine also demonstrated a low order of analgesic and antipyretic efficacy. Evidence from several preliminary testing procedures indicated that cryogenine pretreatment afforded significant protection against serotonin-induced pedal edema in the rat and histamine-induced intradermal wheal responses in the rabbit. Moreover, the results of several in vitro investigations (isolated guinea pig ileum) demonstrated that cryogenine could antagonize several suspected mediators of inflammation

(bradykinin, histamine, serotonin). Partial reversal of histamine antagonism was effected by increasing concentrations of the agonist (15). Some limited specificity for the histaminic receptor was confirmed in subsequent studies (17).

Moreover, vascular smooth muscle constriction produced by oxytocin and kallidin (isolated perfused rabbit ear vein) has been reported to be reversibly inhibited by relatively small concentrations of cryogenine (18). Cryogenine has been reported to lack fibrinolytic activity (15). Recent testing has shown the alkaloid to be ineffective in reversing stress-induced hyperglycemia in rats (19). Unlike reserpine or tetrabenazine, cryogenine does not deplete either central or peripheral tissue levels of norepinephrine and serotonin (20). Histopathologic evaluations of the effects produced by chronically administered cryogenine on various organs and tissues revealed no apparent lesions at antiinflammatory dose levels (15,20). Thus, the antiinflammatory activity ascribed to cryogenine would appear to be unrelated to any systemic toxic manifestation of the drug.

An evaluation of data gathered from previous animal studies reveals that cryogenine incorporates several properties classically inherent to most clinically-employed antiinflammatory agents (sedative, antihistaminic, and low orders of analgesic and antipyretic activity). The present study was undertaken to examine more thoroughly certain

aspects of the pharmacologic nature of cryogenine (other than its psychotropic attributes) and to compare its antiinflammatory potential with known antiinflammatory agents. Hopefully, interrelationships between the effects observed in this investigation could lead to a delineation of the possible mechanisms responsible for the unique antiinflammatory activity attributed to cryogenine.

## MATERIALS AND METHODS

### 1. Adjuvant-Induced Arthritis

Adult female rats of the Sprague-Dawley strain weighing approximately 200 g were employed in all experiments. On arrival, animals were housed in pairs and allowed to acclimate to the new environment for a period of not less than one week prior to experimentation. Ambient temperatures were maintained at 73-76° F. All animals were offered a diet of commercially prepared food pellets[1] and tap water *ad libitum*.

Heat-killed, dessicated *Mycobacterium butyricum*[2] was suspended in light mineral oil (USP) (5mg/ml) with a hand homogenizer[3]. This suspension was sterilized in

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[1] Purina Rat Chow. Crude protein not less than 30.0%, crude fat not less than 4.5%, crude fiber not less than 6.0%, ash not less than 9.0%, plus vitamin and mineral supplement.

[2] Difco Laboratories, Detroit, Michigan. Catalogue number 0640-33.

[3] Kontes glass jacketed tissue homogenizer, Kontes Glass Company, Vineland, New Jersey.

an autoclave (20 pounds pressure for 20 minutes) and subsequently stored in a refrigerator in 4 ml rubber-capped amber serum bottles until needed. Prior to injection, the "adjuvant" was warmed slightly to above room temperature and thoroughly agitated.

The polyarthritic syndrome was induced by a single subcutaneous injection of 0.05 ml of the finely triturated suspension of M. butyricum into the subplantar surface of the left hind paw. Rats were first lightly anesthetized with sodium pentobarbital (30 mg/Kg i.p.) to ensure precision in the placement of the adjuvant injection. Injections were made using 1 inch 25 gauge sterile disposable needles[1] adapted to a 2.0 ml capacity micrometer syringe[2]. To avoid external leakage of the adjuvant mixture, the hypodermic needle was inserted through the radial foot pad and directed medially into the metatarsal region, keeping the needle channel as near to the skin surface as possible to minimize the possibility of vascular involvement.

Prior to injection of the adjuvant, the rats were randomly divided into groups of 10 animals each. Each study included 2 control groups: a "positive control" group which received adjuvant but no drug treatment, and a "negative control group" of 10 animals receiving neither

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[1] Becton, Dickinson and Company, Rutherford, New Jersey.
[2] Roger Gilmont Instruments, Inc. 1 Great Neck Road, Great Neck, New York. Catalogue number S 1200.

adjuvant nor drug treatment. For each drug evaluation, an additional group of 10 animals was utilized.

All drugs undergoing test were dissolved or suspended in an aqueous solution of 0.25% w/v agar and administered by gastric intubation in a dosage volume of 0.5 ml per 100 g body weight. Oral dosing was selected in order to minimize parenteral counter-irritant effects (25). Fresh drug suspensions were prepared daily.

The administration of all test drugs was initiated 1 day preceding adjuvant injection and continued daily for 21 days (days "0" through "20"). Control groups received only the requisite volume of the agar vehicle. To determine the progress of the inflammatory response in the polyarthritic rats, several metameters were collected utilizing the following methods.

Plethysmographic Determination of Hind Paw Volumes.— Both injected and contralateral paw volumes were measured plethysmographically by displacement of mercury using a procedure similar to that described by Winder et al. (26). Hind paw volume measured to an india ink line across the lateral malleolus was determined by the displacement of mercury in a vertically uniform plexiglas vessel (diameter = 2.5 cm, height = 6.0 cm). The resulting change in pressure was determined with a pressure transducer[1]

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[1] Statham pressure transducer P23BB. Statham Laboratories Inc., Hato Rey, Puerto Rico.

coupled to a direct writing recorder through a strain gauge coupler[1]. A resistor-capacitor network[2] was introduced into the circuit to filter out high frequency signals usually encountered at the sensitivity levels required. Volume displacement was calibrated by periodically introducing a graduated aluminum rod of uniform diameter scored at 0.5 ml intervals into the mercury-containing vessel. The system was equilibrated to atmospheric pressure immediately prior to each series of measurements (Figure 2.). The therapeutic effects of the various drug treatments were measured in a manner similar to that originally described by Newbold (27) as percentage inhibition of increased foot volume. The following formula was employed:

$$\text{Percentage inhibition} = 100\left[1-\frac{(a-x)}{(b-y)}\right]$$

Where y = mean foot volume of control rats immediately prior to adjuvant injection, b = mean foot volume of control rats on a particular day, x = mean foot volume of drug-treated rats immediately prior to adjuvant injection, and a = mean foot volume of drug-treated rats on a particular day. Paw volumes were determined every third day commencing on day "0" through day "30."

Arthritic Index.-- A subjective scoring system for assessing the progressive development of the induced

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[1] Type 9872 strain gage coupler. Beckman Instruments, Inc. 3900 River Road, Schiller Park, Illinois.

[2] Modification of the Dynograph was instituted by Mr. Frank A. McKenna of Beckman Instruments, Inc.

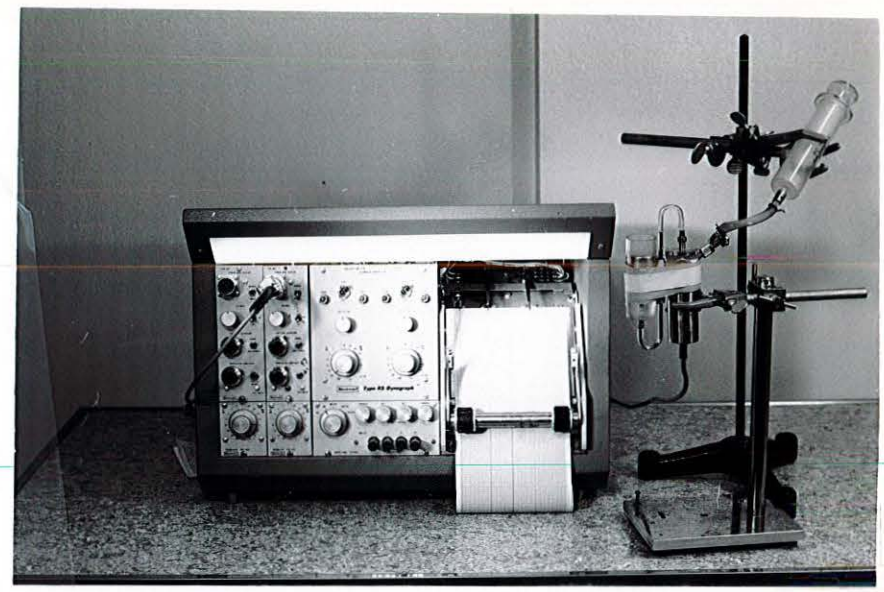

Figure 2.-- Recording system employed in foot volume determinations

arthritic syndrome was employed (28). The severity of the arthritic lesions in each of the four legs was assigned a value of 0 to 4 according to the extent and degree of involvement. Criteria for grading were the severity and extent of edema and erythema of the periarticular tissue, and the enlargement, distortion and ankylosis of the joints. Therefore, the arthritic score for any one animal could range from 0 to a maximum score of 16. The arthritic index is the mean of individual scores for a group. Various lesions associated with adjuvant-induced arthritis are illustrated in Figures 3. and 4. Scoring was visually assessed every third day following adjuvant injection (day "3" to "30"). The percent inhibition was determined by comparing the index of the drug-treated group to that of a positive vehicle control group.

Impairment of Body Growth.-- To assess the general state of health of animals during the course of the polyarthritic disease, mean body weights were determined for both control and drug-treated groups initially and at 3 day intervals during the 30 day observation period. In addition, the adrenal glands, spleen and thymus were removed and weighed from control rats and from drug-treated rats sacrificed 15, 21 and 30 days after the injection of adjuvant.

Effect of Cryogenine on Previously Established Arthritis.-- Arthritis was induced in a surplus number of

Figure 3.-- Secondary Lesions in Polyarthritic Rats (Left - Positive Control, Right - Negative Control.)

A. Early signs of ear lesions, Day "15"

B. Swelling of front paws, nose and head area, Day "21"

C. Nodular swelling of the tail and hind paws, Day "30"

Figure 4.-- Secondary lesions in polyarthritic rats (Left - positive control, Right - negative control)

positive control rats by methods described above. On the twenty-first day after adjuvant injection (when the arthritic syndrome was fully developed), rats with mean paw volumes exceeding 2.50 ml were selected and placed into two experimental groups of 4 animals each. One group was treated daily with cryogenine acetate (100 mg/Kg in aqueous 0.25% agar, orally) for 7 days, while the other group received an equivalent volume of the agar vehicle only (5 ml/Kg). Paw volumes and arthritic index determinations were assessed daily.

Serum Turbidity in Adjuvant-Injected Rats.-- The polyarthritic disease was established in experimental groups of rats as described above. Serum turbidity levels were measured in 8 negative and positive control animals (4 agar-treated and 4 nontreated adjuvant-injected animals). In addition, serum turbidity was determined in rats treated with cryogenine and the other selected antiinflammatory agents orally for 21 days. Serum turbidity determinations were made 20 days following adjuvant administration using a procedure similar to that described by Piliero and Colombo (29). Rats, lightly anesthetized with ether, were exsanguinated by cardiac puncture. To 0.1 ml of nonhemolyzed serum, 2.9 ml of 1/15 M Sorensen phosphate buffer[1] in saline at pH 5.2

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[1] Composition of buffer: 2 ml 1/15 molar monopotassium phosphate + 98 ml 1/15 molar disodium phosphate.

were added and gently agitated. The mixture was allowed to stabilize at room temperature for 15 minutes. The mixture was then heated in a constant temperature water bath at 69° C for 30 minutes at which time the samples were immediately cooled in an ice bath and read in a spectrophotometer[1] at a wavelength of 645 mμ. Changes in turbidity were based on the degree of absorbancy.

Hematological Studies in Adjuvant-Injected Rats.--- During the course of the adjuvant-induced arthritic syndrome, hematologic studies were conducted in animals from both positive and negative control groups (adjuvant-injected agar-treated and noninjected agar-treated groups, respectively) and in the arthritic groups treated with cryogenine and the other selected antiinflammatory drugs. Differential white blood cell counts from smears stained with tetrachrome[2], and total white blood cell counts determined with a Coulter counter[3] were recorded on days "6", "15", "21" and "30" from blood obtained from rats anesthetized lightly with ether and exsanguinated by cardiac puncture. Erythrocyte sedimentation rates were also determined in all groups. A 0.4 ml volume of a 15% EDTA solution, employed as an anticoagulant, was added to each 2 ml sample of cardiac blood. Sedimentation rates were measured as mm fall per 2 hours.

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[1] Bausch & Lomb "Spectronic 20".

[2] Tetrachrome stain #637. Allied Chemical Corp. New York, New York.

[3] Coulter Counter, Model F. Coulter Electronics, Inc. Hialeah, Florida.

2. Ultraviolet-Induced Erythema

Healthy adult male albino guinea pigs of the Hartley strain weighing 300-400 g were employed throughout these studies. On arrival, the animals were housed in pairs in medium-sized wire floor cages. Purina Guinea Pig Chow[1] and water (supplemented with 0.25 mg/ml ascorbic acid) were supplied ad libitum. Ambient room temperature was maintained at 73-76° F. Sixteen hours prior to the standardized ultraviolet exposure, the ventro-lateral surface of a flank of each animal was depilatated with a commercially available calcium thioglycolate preparation[2]. Following depilatation, animals were randomly assigned into experimental groups and sequentially placed into individual cages to allow for a constant length of time to elapse between depilatation and ultraviolet exposure. Food was also withdrawn from the animals at this time. All drugs were administered orally using standard feeding needles attached to 2.5 ml glass syringes. Drugs were either administered as solutions or finely triturated suspensions in 0.25% aqueous agar[3]. The constant volume of injection was 0.5 ml per 100 g body weight. Experimental control animals received the agar vehicle only. To facilitate dosing, the guinea pigs were

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[1] Crude protein not less than 22.0%, crude fat not less than 4.0%, crude fiber not more than 18.0%, added minerals not less than 3.5%.

[2] Neet®; Whitehall Laboratories, Inc., New York, N.Y. 10017.

[3] Difco Bacto-agar, Difco Laboratories, Detroit, Michigan.

restrained by a folded towel wrapped around the forelegs and body. Each dose level of a test drug was administered to an experimental group consisting of 5 animals. Each dose was administered in two equal parts separated by a one hour interval. Immediately following the second dose, the depilatated surface of each animal was exposed to the standardized ultraviolet irradiation for a period of 70 seconds.

The apparatus employed for inducing the erythemic response is illustrated in Figure 5 . A 3/8 inch thick, 20 inch square of opaque plexiglas was positioned directly above an opaque shutter. The ultraviolet source, 12.5 cm below, consisted of a Hanovia high pressure quartz mercury vapor lamp (450 Watt)[1] mounted in an Alzak aluminum reflector hood assembly[1]. The base of the reflector housing was secured to an adjustable support platform[2] to facilitate the positioning of the lamp. The radiation directed upward from the reflector was passed through a 6½ inch square molded Vycor heat absorbing filter[1] positioned above the ultraviolet light source and directly below the shutter. A milled-out area in the plexiglas (1 x 3 inch) accommodated an opaque plastic plate into which three equally-spaced 6½ mm diameter holes

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[1] Supplied by Hanovia Lamp Division, 100 Chesnut Street, Newark, New Jersey 07105.

[2] Precision Scientific Company, Chicago, Illinois. "Big Jack" support platform.

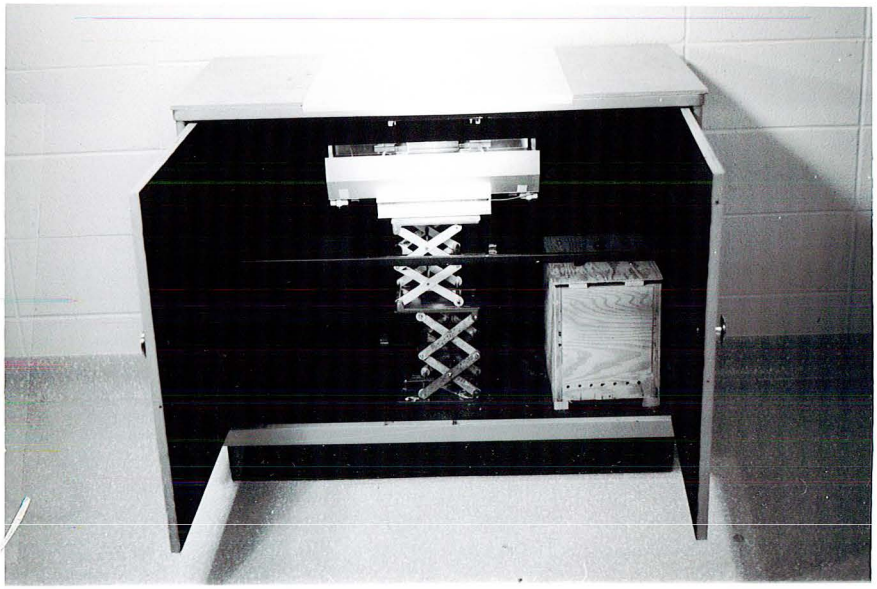

Figure 5.-- Apparatus employed in ultraviolet induced-erythema experiments

(each separated by a distance of 7 mm) were drilled. Each animal was positioned and held directly over the plastic plate. The duration of ultraviolet exposure was automatically controlled by the activation of an electric interval timer[1] which energized a small solenoid attached to the shutter. The lamp was allowed a "warm up" period of 30 minutes prior to use.

The effectiveness of the drugs in delaying or suppressing the ultraviolet-induced erythema was evaluated using a modification of the methods described by Brittain and Spencer (30) and Winder et al. (31). The average intensity of the resulting erythematous circles as well as the appearance of the non-exposed depilatated skin varies markedly among similarly treated animals. Rather than attempting to quantitate the degree of "redness" or its contrast relative to the unexposed areas of depilatated skin, a rating system similar to that described by Winder et al. was employed based on the "definiteness" or "indefiniteness" of developing erythema. An exposed spot demonstrating no apparent erythema = 0; a spot with slight erythema, and poorly defined borders = 1; a spot showing moderate erythema and defined borders = 2; a full circle of marked erythema = 3. The average "definiteness" of the three resulting erythematous circles was estimated at 1 hour intervals for a period of 3 hours. The average

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[1] Singer Industrial Timer Corp. Parsipany, New Jersey. Master Time-O-Lite.

score was determined for control animals and for the experimental animals at each dose level of the test compounds. The responses of each experimental group of 5 animals were summed (group inflammatory score) and plotted against time. The area under the time-responses curve represents the level of inflammation over the 3 hour test period. The area beneath the curve, plotted for each dose level of a test drug, was determined and expressed as a percentage of the response for untreated control animals. The protective efficacy of drugs against ultraviolet-induced erythema was determined from log-dose response curves based on tests at at least three equally-spaced, log-dose levels per compound. The percent inhibition of the control group response produced by a test compound was plotted against the corresponding dose level on probability-logarithmic graph paper[1]. The dose necessary to reduce the value of the control response by half ($ED_{50}$) was graphically determined from the resultant linear expression.

3. Acute Cardiovascular Interactions

Healthy adult male rats of the Sprague-Dawley strain were anesthetized with Dial-Urethane[2] (0.1 ml per 100 g body weight) administered intraperitoneally. Following tracheal intubation, the left common carotid artery was

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[1] Keuffell & Esser Company. #359-24.
[2] Dial with Urethane Solution, Ciba Pharmaceutical Co., Summit, New Jersey.

isolated and carefully separated from the vago-sympathetic trunk. A polyethylene cannula (P.E. 50) was inserted into the artery and blood pressure was monitored by means of a Statham pressure transducer[1] employed in conjunction with a strain gauge coupler[2] and recorded on a Beckman Dynograph. A short catheter (P.E. 10) was inserted into the left femoral vein for the intravenous injection of all drugs. The constant volume of injection was 0.1 ml followed by a 0.1 ml wash of 0.9% sodium chloride solution to insure accuracy of dosage. All drugs were dissolved in 0.9% saline. The following drugs were used as "challenge" agents: acetylcholine chloride, epinephrine hydrochloride, bradykinin, serotonin creatinine sulfate, and histamine dihydrochloride. After determination of standardized responses produced by intravenous injections (one second duration) of the test challenges, cryogenine acetate was administered in cumulative doses at 10 minute intervals followed by the test challenges 2 minutes later. Mean arterial blood pressure was determined prior to and immediately following each drug administration.

The effects of cryogenine on the test challenge responses were estimated by comparisons with the standardized responses evoked by the test challenges before

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[1] Statham pressure transducer P23 AC. Statham Laboratories Inc., Hato Rey, Puerto Rico.

[2] Type 9872 strain gage coupler, Beckman Instruments Inc., 3900 River Road, Schiller Park, Illinois.

cryogenine administration.

4. Drug-Induced Bronchoconstriction

To test antagonism of bronchoconstriction induced by bradykinin, histamine and 5-hydroxytryptamine, guinea pigs of either sex (500-1200 g) were anesthetized with urethane (1.5-2.0 g/Kg i.p.). The resistance of the guinea pig lungs to inflation, in vivo, was determined by a modification of methods originally described by Konzett and Rossler (32). The trachea was cannulated and the lungs inflated with a small animal respirator[1] operating at a rate of 66 strokes per minute in a partially closed system at a constant stroke volume (3-7 cc). The stroke volume was adjusted at the beginning of each experiment to produce a minimal overflow volume of air through a sidearm in the tracheal cannula. Bronchoconstriction was assessed from the increase in the air overflow which was determined by means of a small volumetric transducer[2] employed in conjunction with a polygraph recorder[3].

All bronchoconstrictor drugs were dissolved in a solution of 0.9% sodium chloride and were injected in a constant dose volume of 0.1 ml through a short polyethylene

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[1] Miniature Ideal Pump Assembly, Model # 5119/04 manufactured by C.F. Palmer Ltd. Carlisle Road, London, England.

[2] Statham pressure transducer # PT-5. Statham Laboratories Inc., Hato Rey, Puerto Rico.

[3] Grass Model 7 polygraph, Grass Instrument Co. Quincy Mass. 02169.

catheter (P.E. 50) inserted into the left external jugular vein. Drug injections were immediately followed by a 0.1 ml 0.9% saline wash. The following drugs were used: histamine dihydrochloride, 5-hydroxytryptamine (serotonin creatinine sulfate, 5-HT), synthetic bradykinin[1] and cryogenine acetate. All doses are expressed as the base form of the drug.

The effect of cryogenine on bradykinin-, histamine-, or serotonin-induced bronchoconstriction was estimated from comparisons between the response evoked by these agents when administered 5 minutes prior to and at 5 or 10 minute intervals following intravenous administration of cryogenine.

5. Ganglionic Interactions

A series of experiments were performed on the superior cervical ganglia of young adult (250 g) Wistar rats (Charles River). The surgical and recording procedures employed in this study were similar to those described by Hancock et al. (33,34). Rats of either sex were anesthetized with a mixture[2] of allobarbital (100 mg/Kg), urethane (400 mg/Kg) and monoethylurea (400 mg/Kg) administered intaperitoneally. Following intubation of the trachea, the left superior cervical ganglion was

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[1] Synthetic Bradykinin (BRS 640) Sandoz Pharmaceuticals, Hanover, New Jersey.

[2] Dial with Urethane Solution. Ciba Pharmaceutical Co. Summit, New Jersey.

exposed by retraction of the upper portions of the trachea and esophagus and reflection of the longus capitus muscle. The postganglionic nerve (external carotid) was identified, ligated and sectioned at its point of contact with the external carotid artery. The cervical sympathetic trunk was dissected free from the vagus nerve and common carotid artery and sectioned at a point approximately 0.5 cm above the clavicle. All major branches of the common carotid artery, except those supplying the ganglion were tied. The external carotid artery was ligated at the level of the occipital artery. Similarly, the internal carotid artery was tied approximately 5 mm cephalad to the superior cervical ganglion. A deep cervical well was formed by tying skin flaps to the supporting metal framework. The exposed area was covered with mineral oil. Supramaximal stimuli of 0.2 msec duration were applied to the cervical sympathetic trunk by means of a square wave generator[1], the output of which was passed through a stimulus isolation unit[1] to bipolar platinum electrodes. Unless indicated otherwise, the sympathetic trunk was stimulated at a rate of 0.3 cps. Ganglionic potentials evoked by drugs or preganglionic stimulation were recorded from the surface of the ganglion by means of silver-silver chloride bipolar electrodes. One electrode was placed in direct contact

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[1] Stimulus isolation unit Model SIU 4, Square wave stimulator Model S 4. Grass Instrument Co. Quincy, Mass.

with the surface of the ganglion, the other through a saline saturated silk ligature on the crushed end of the postganglionic nerve. The evoked potentials were amplified by a resistance-coupled preamplifier[1] and visualized on an oscilloscope[2]. Drug-evoked asynchronous postganglionic activity was recorded by means of bipolar platinum electrodes placed in a similar manner to that described above and amplified through a capacitance-coupled circuit. All drugs were administered intraarterially through a 27 gauge needle inserted into the common carotid artery and secured to the supporting framework. The constant volume of injection was 0.05 ml. All drugs were dissolved in a solution of 0.9% sodium chloride. Clotting in the needle was prevented by the prior administration of heparin (400 U/Kg, intraarterially). The following drugs were used: acetylcholine chloride, methacholine chloride, oxotremorine picrolonate, atropine sulfate, potassium chloride, hexamethonium chloride, epinephrine bitartrate, levarterenol bitartrate, d,l-isoproterenol hydrochloride, serotonin creatinine sulfate (5-HT), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), tetramethylammonium chloride (TMA), physostigmine salicylate, 4-(m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) and cryogenine acetate.

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[1] Grass Model P 6 preamplifier. Grass Instrument Co. Quincy, Mass.

[2] Tektronix, Inc. Model 502A.

With the exception of cryogenine, all doses refer to the salt form of the drug.

## RESULTS

### 1. Adjuvant-Induced Arthritis

Plethysmographic Determination of Hind Paw Volumes.--
The single injection of heat-killed *Mycobacterium butyricum* into the plantar surface of the right hind paw produced a systemic arthritic syndrome in all control group animals. Marked inflammatory swelling was noted in the injected paw (primary lesion) within a period of 2 hours following adjuvant injection and this swelling continued to increase for 8 or 9 days. After a plateau stage, a further increase in paw volume occurred and persisted for the remainder of the 30 day observation period. Secondary lesions, associated with the acute phase of the disease and evidenced as swelling in the contralateral (non-injected) paw, became apparent 13 to 17 days after adjuvant injection and progressively increased in severity for the next 12 to 13 days. The drug treatments employed from days "0" through "20" are listed in Table I. The therapeutic efficacy of these

Table I.-- Drug Treatments Employed in Adjuvant-Induced Arthritis[1]

| Treatment Group | Oral Dosage, mg./Kg./Day |
|---|---|
| Negative Control | --- |
| Positive Control | --- |
| Cryogenine | 100 |
| Phenylbutazone | 100 |
| Mefenamic Acid | 25 |
| Indomethacin | 1.0 |
| Paramethasone | 0.5 |
| Hydrocortisone | 10 |
| 6-mercaptopurine | 2.0 |

[1] All drugs administered for 21 days beginning one day prior to adjuvant administration.

agents was assessed at three day intervals during the course of the adjuvant disease. Foot volume determinations indicated that all of the test agents inhibited the inflammatory swelling to varying degrees; however, none prevented the inflammation seen immediately following the adjuvant injection. The therapeutic effects produced by the test compounds on the primary and secondary lesions (injected and contralateral foot volumes, respectively) are graphically represented in Figure 6. through Figure 12. Inhibition of edema in the injected foot was achieved with indomethacin, phenylbutazone, hydrocortisone and cryogenine at the dose levels employed. Secondary lesions in contralateral (non-injected) paws were significantly suppressed by all agents tested during the period of therapeutic treatment. After cessation of treatment these beneficial effects persisted -- especially in animals treated with paramethasone and to a lesser extent with 6-mercaptopurine, phenylbutazone and cryogenine. Contralateral feet of paramethasone-treated rats appeared essentially normal and equivalent to non-injected control animals at the termination of the 30 day observation period. The therapeutic effects elicited by daily dosage of indomethacin, hydrocortisone and mefenamic acid however, were not sustained after cessation of dosage. This lack of protection was reflected by a significant increase in paw volume soon after drug treatment ended. A summary of test drug effects on the course of adjuvant-induced arthritis is

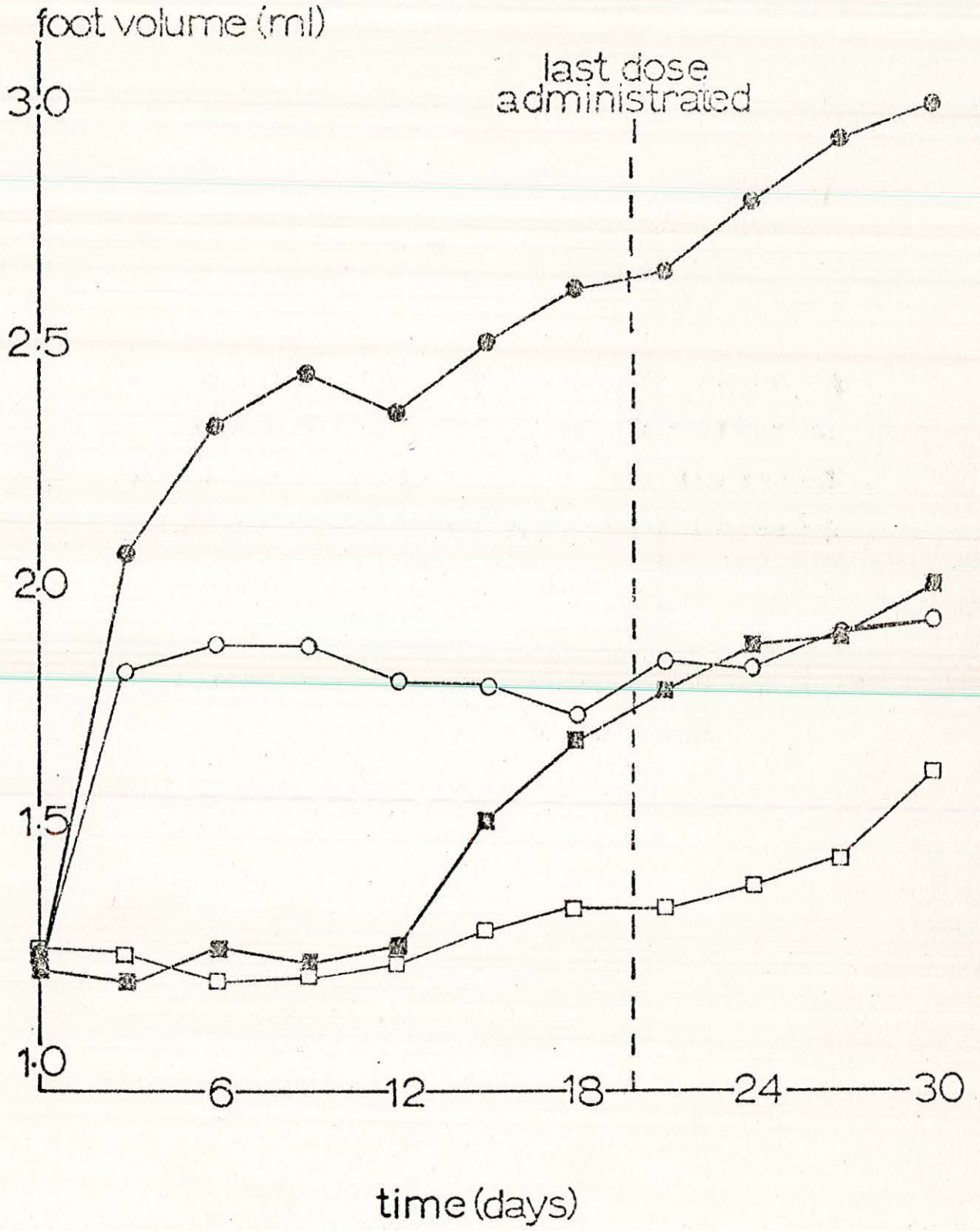
 = injected foot volume of positive control animals

○ = injected foot volume of cryogenine-treated animals

 = contralateral foot volume of positive control animals

□ = contralateral foot volume of cryogenine-treated animals

Figure 6 — Adjuvant-induced arthritis in rats treated with cryogenine (100 mg./Kg.)


foot volume (ml)
last dose
administrated
3·0
2·5
2·0
1·5
1·0
6
12
18
24
30
time (days)

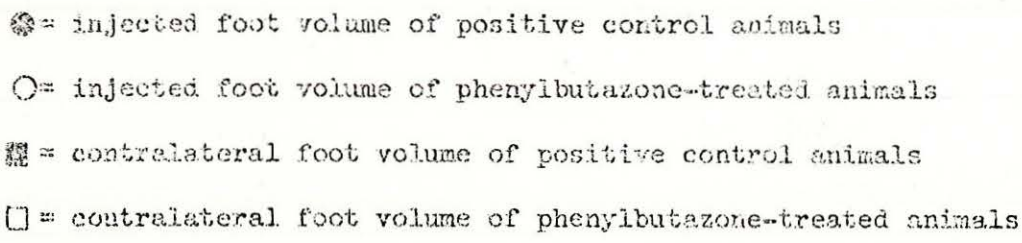
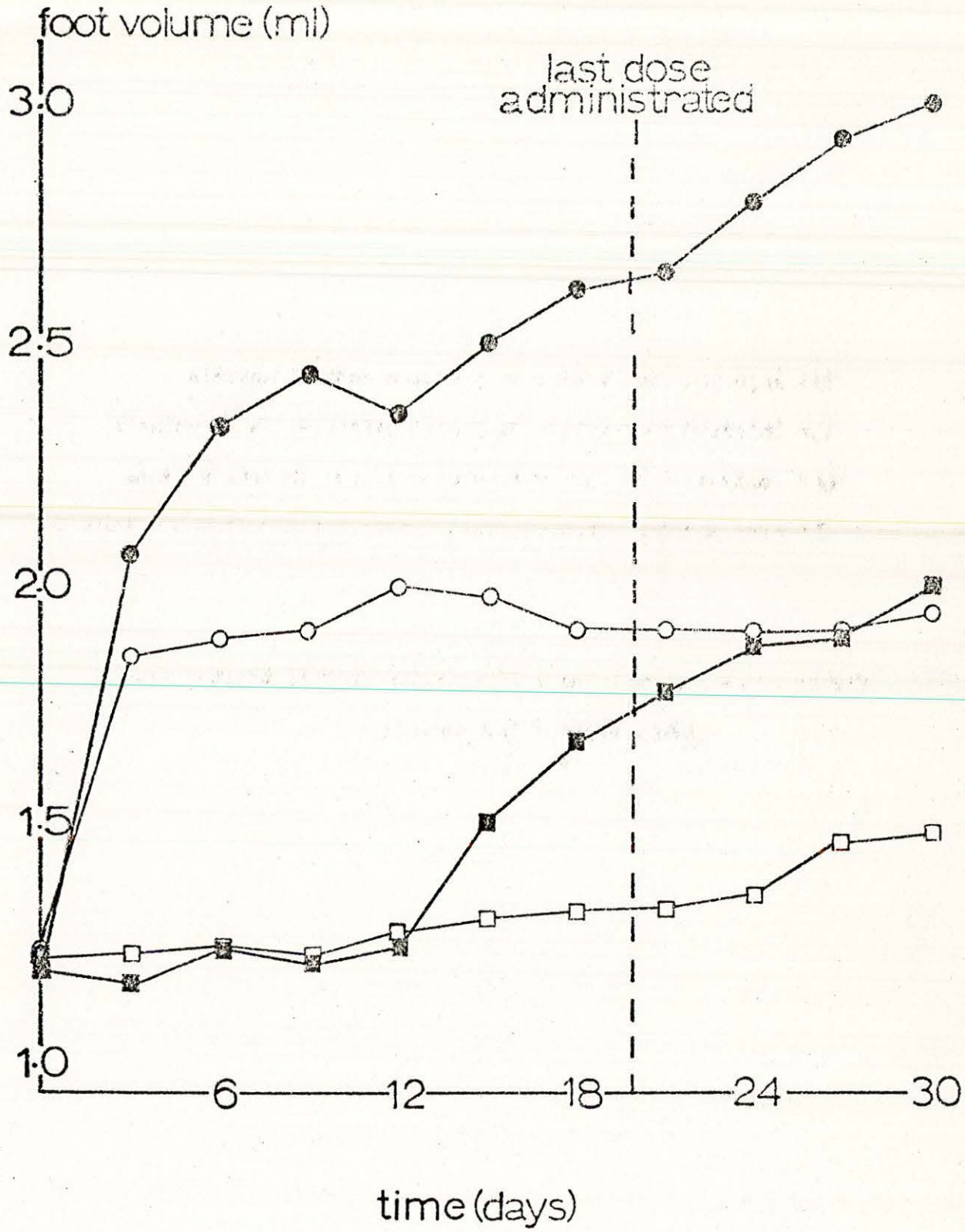



Figure 7.-- Adjuvant-induced arthritis in rats treated with phenylbutazone (100 mg./Kg.)


foot volume (ml)
3·0
2·5
2·0
1·5
1·0
last dose
administrated
6
12
18
24
30
time (days)

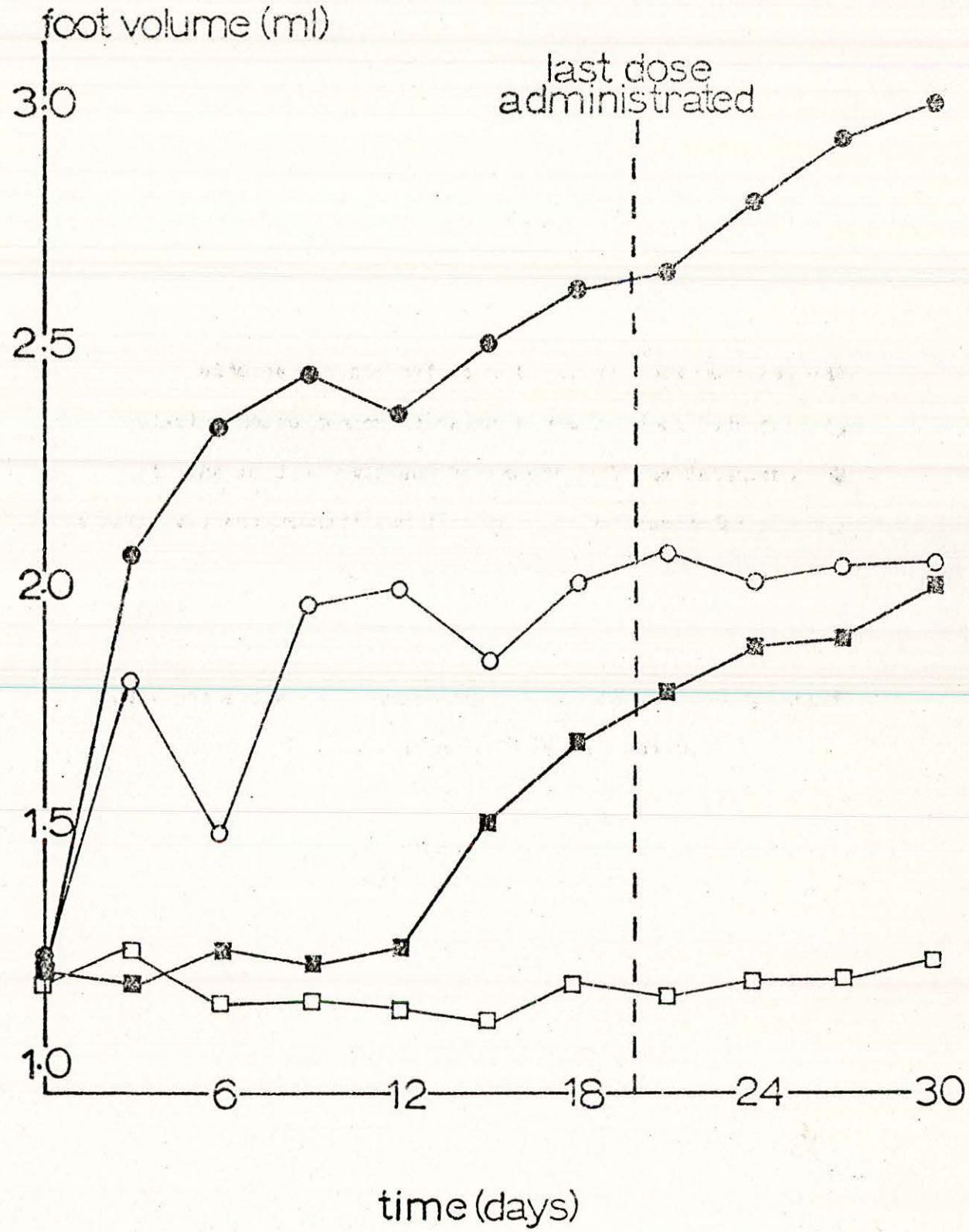
● = injected foot volume of positive control animals

○ = injected foot volume of paramethasone-treated animals

▪ = contralateral foot volume of positive control animals

□ = contralateral foot volume of paramethasone-treated animals

Figure 8.-- Adjuvant-induced arthritis in rats treated with paramethasone (0.5 mg./Kg.)


foot volume (ml)
last dose
administrated
3·0
2·5
2·0
1·5
1·0
6
12
18
24
30
time (days)

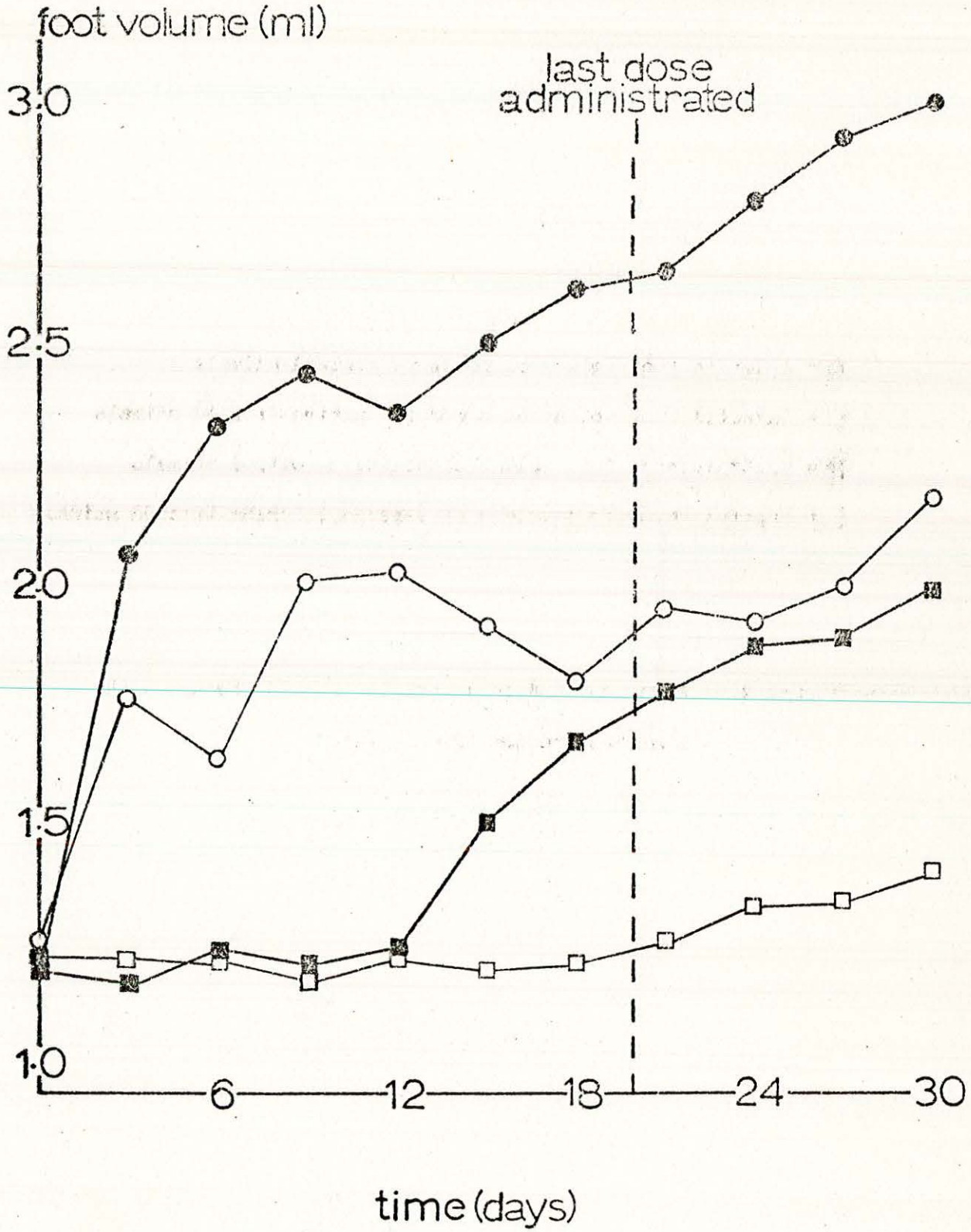
● = injected foot volume of positive control animals

○ = injected foot volume of 6-mercaptopurine-treated animals

■ = contralateral foot volume of positive control animals

□ = contralateral foot volume of 6-mercaptopurine-treated animals

Figure 9.-- Adjuvant-induced arthritis in rats treated with 6-mercaptopurine (2.0 mg./Kg.)


foot volume (ml)
last dose
administrated
3·0
2·5
2·0
1·5
1·0
6
12
18
24
30
time (days)

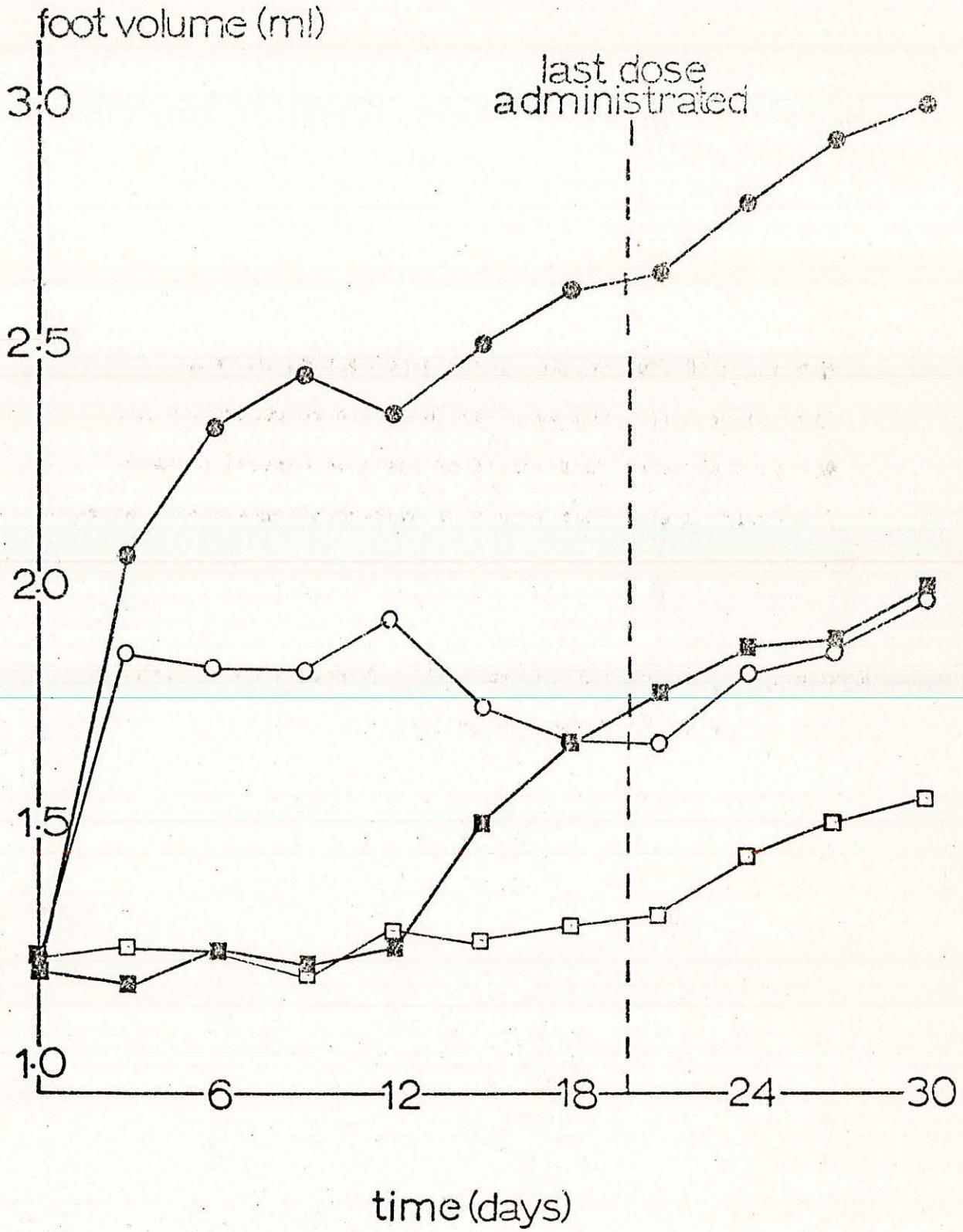
● = injected foot volume of positive control animals

○ = injected foot volume of hydrocortisone-treated animals

■ = contralateral foot volume of positive control animals

□ = contralateral foot volume of hydrocortisone-treated animals

Figure 10.-- Adjuvant-induced arthritis in rats treated with hydrocortisone (10 mg./Kg.)


foot volume (ml)
last dose
administrated
3·0
2·5
2·0
1·5
1·0
6
12
18
24
30
time (days)

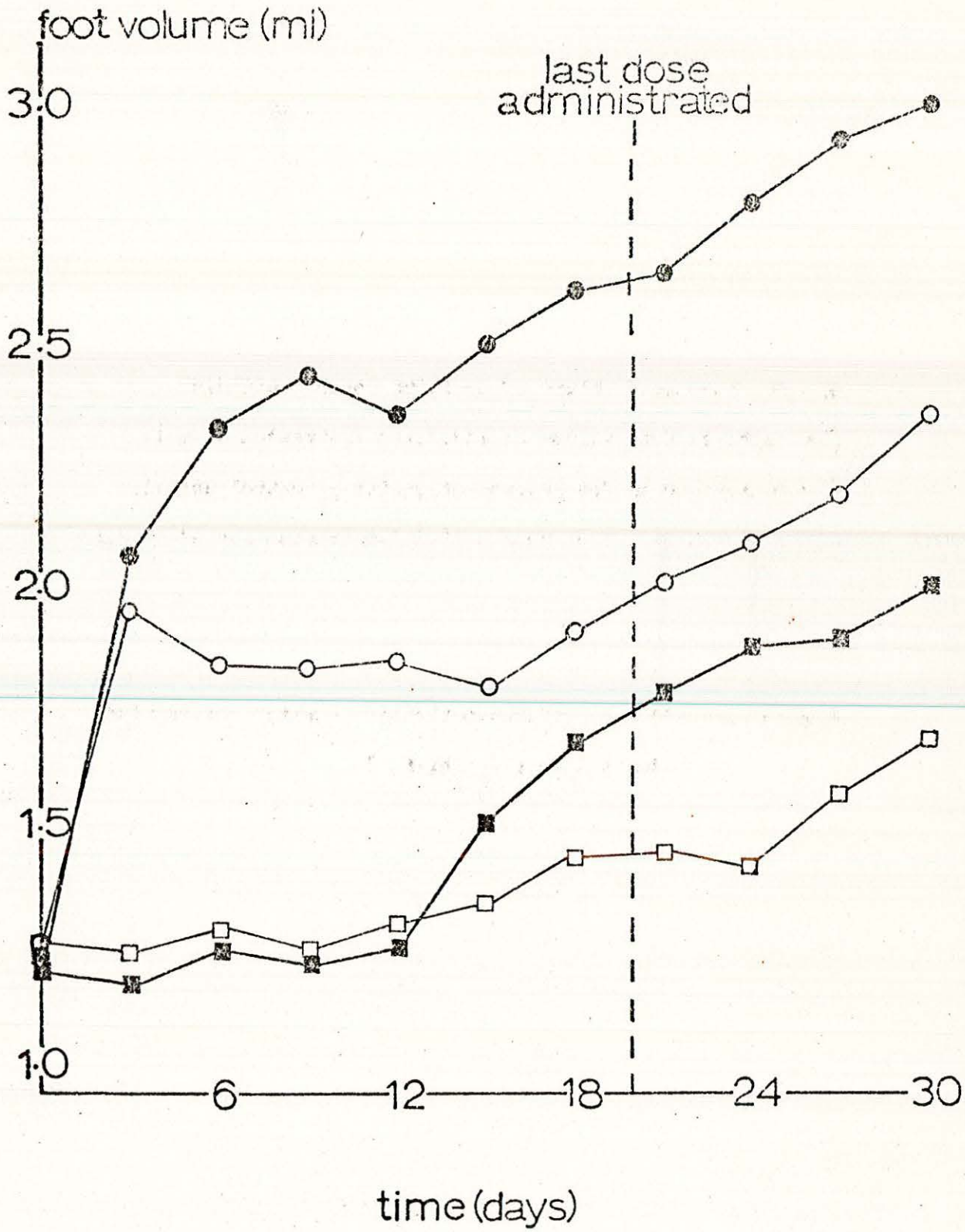
● = injected foot volume of positive control animals

○ = injected foot volume of indomethacin-treated animals

▩ = contralateral foot volume of positive control animals

□ = contralateral foot volume of indomethacin-treated animals

Figure 11.-- Adjuvant-induced arthritis in rats treated with indomethacin (1.0 mg./Kg.)


foot volume (ml)
last dose
administrated
3·0
2·5
2·0
1·5
1·0
6
12
18
24
30
time (days)

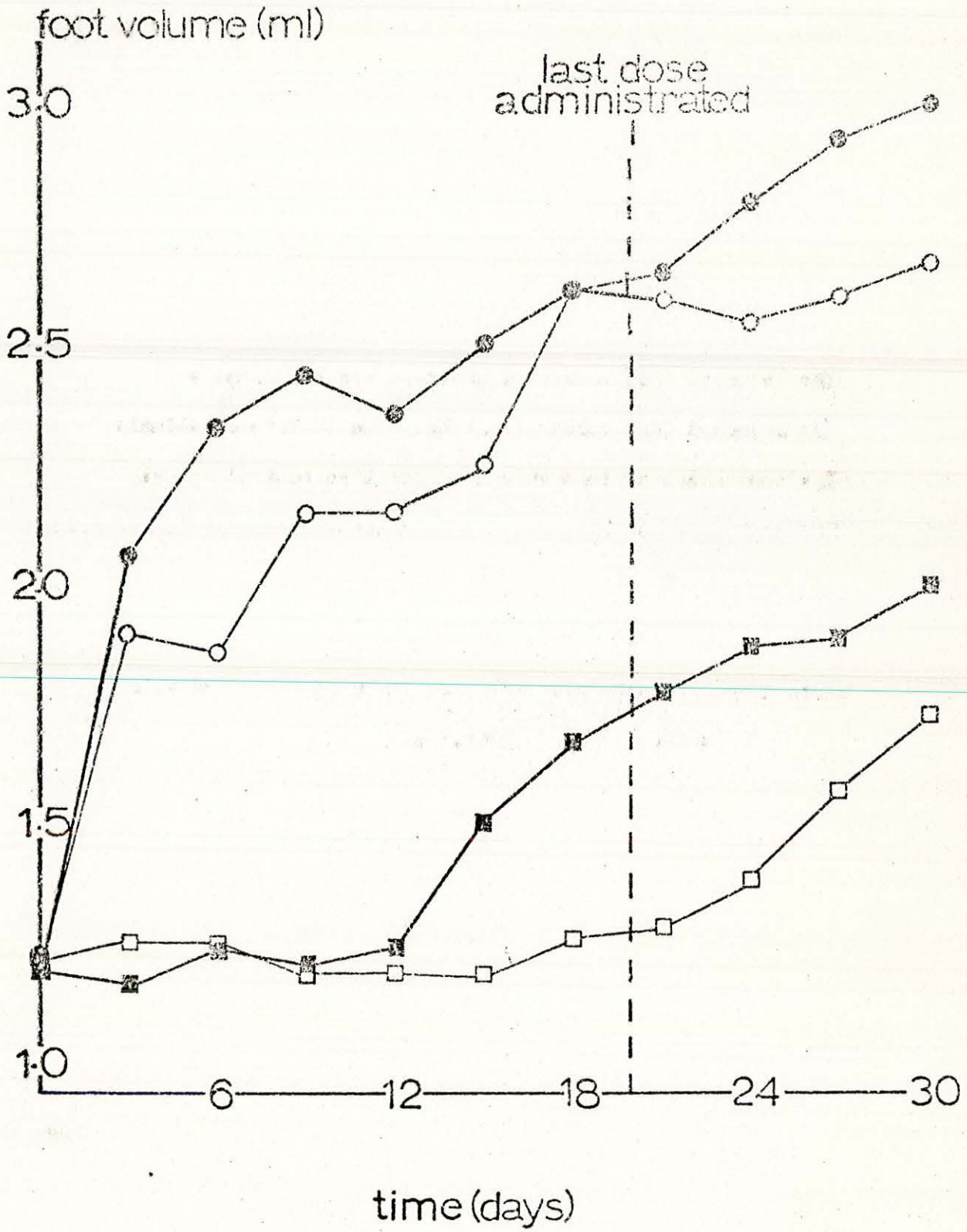
● = injected foot volume of positive control animals

○ = injected foot volume of mefenamic acid-treated animals

▩ = contralateral foot volume of positive control animals

□ = contralateral foot volume of mefenamic acid-treated animals

Figure 12.-- Adjuvant-induced arthritis in rats treated with mefenamic acid (25mg./Kg.)


foot volume (ml)
3·0
2·5
2·0
1·5
1·0
last dose
administrated
6
12
18
24
30
time (days)

presented in Tables II, III, and Tables IV, V.

Arthritic Index.--- The local inflammatory response elicited by the subplantar injection of mycobacterial adjuvant occurred almost immediately after administration in all control group animals. The initial edematous reaction plateaued usually within 3 or 4 days; however, a moderate level of swelling continued to persists until Day "13" to Day "15", at which time, the beginning manifestation of secondary lesions became evident. These were noted as increased vascularity of the ears, nodular lesions of the nose and tail, and swelling of front and contralateral (noninjected) hind paws.. Ear lesions, which were first observed as areas of apparent capillary dilation, developed into inflammed nodules. Similarly, early lesions of the tail increased in severity and caused a marked increase in tail thickness. Secondary lesions of the hind feet were observed initially as a diffuse erythematous swelling of the dorsum of the tarsal region and ankle joints with attendant inflammed fusiform swelling of one or more digits of the paw. The disseminated arthritis developed at a relatively slow rate, but progressively increased in severity over the 30 day observation period.

Figure 13. to Figure 19. indicate the therapeutic efficacy of cryogenine and the selected antiinflammatory agents on the course and development of adjuvant arthritis as

Table II.-- Effects of Drug Treatments on Injected-Paw Swelling in Adjuvant-Induced Arthritis

| | Foot Volume, ml. ±S.E. | | | | | |
|---|---|---|---|---|---|---|
| Treatment Group | Day 3 | Day 9 | Day 15 | Day 21 | Day 24 | Day 30 |
| Negative Control | 1.21 ±0.02 | 1.24 ±0.03 | 1.26 ±0.04 | 1.26 ±0.04 | 1.28 ±0.06 | 1.27 ±0.04 |
| Positive Control | 2.07 ±0.06 | 2.44 ±0.11 | 2.51 ±0.19 | 2.65 ±0.15 | 2.85 ±0.19 | 3.03 ±0.21 |
| Cryogenine | 1.84 ±0.07 | 1.89 ±0.07 | 1.80 ±0.08 | 1.85 ±0.07 | 1.84 ±0.09 | 1.94 ±0.09 |
| Phenylbutazone | 1.91 ±0.04 | 1.92 ±0.04 | 1.99 ±0.06 | 1.92 ±0.06 | 1.94 ±0.05 | 1.96 ±0.05 |
| Mefenamic Acid | 1.91 ±0.05[a] | 2.16 ±0.04 | 2.26 ±0.10[a] | 2.61 ±0.08[a] | 2.57 ±0.06[a] | 2.69 ±0.08[a] |
| Indomethacin | 1.97 ±0.08[a] | 1.89 ±0.07 | 2.08 ±0.05 | 2.02 ±0.13 | 2.10 ±0.18 | 2.37 ±0.18 |
| Paramethasone | 1.81 ±0.05 | 1.97 ±0.10 | 1.86 ±0.07 | 2.08 ±0.05 | 2.02 ±0.05 | 2.06 ±0.04 |
| Hydrocortisone | 1.87 ±0.05 | 1.83 ±0.03 | 1.75 ±0.05 | 1.67 ±0.06 | 1.82 ±0.07 | 1.99 ±0.09 |
| 6-mercaptopurine | 1.77 ±0.04 | 2.02 ±0.05 | 1.93 ±0.07 | 1.96 ±0.07 | 1.94 ±0.08 | 2.20 ±0.06 |

[a] Not significantly different from Positive Control group values ($P = 0.05$)

Table III.-- Effects of Drug Treatments on Injected-Paw Swelling in Adjuvant-Induced Arthritis

| Treatment Group | Percent Inhibition of Increased Foot Volume | | | | | |
|---|---|---|---|---|---|---|
| | Day 3 | Day 9 | Day 15 | Day 21 | Day 24 | Day 30 |
| Negative Control | -- | -- | -- | -- | -- | -- |
| Positive Control | -- | -- | -- | -- | -- | -- |
| Cryogenine | 26.2 | 44.6 | 54.7 | 55.6 | 61.7 | 60.0 |
| Phenylbutazone | 21.4 | 44.6 | 42.2 | 52.8 | 57.4 | 61.1 |
| Mefenamic Acid | 17.9 | 22.3 | 18.8 | 2.1 | 16.7 | 18.3 |
| Indomethacin | 15.4 | 52.1 | 57.8 | 46.5 | 48.1 | 38.3 |
| Paramethasone | 27.4 | 36.4 | 48.4 | 38.0 | 49.4 | 52.2 |
| Hydrocortisone | 21.4 | 48.8 | 57.8 | 67.6 | 62.3 | 56.7 |
| 6-mercaptopurine | 39.3 | 37.2 | 47.7 | 50.7 | 58.0 | 47.8 |

Table IV.-- Effects of Drug Treatments on Non-Injected-Paw Swelling in Adjuvant-Induced Arthritis

| | Foot Volume, ml. ±S.E. | | | | | |
|---|---|---|---|---|---|---|
| Treatment Group | Day 15 | Day 18 | Day 21 | Day 24 | Day 27 | Day 30 |
| Negative Control | 1.19 ±0.02 | 1.22 ±0.02 | 1.24 ±0.04 | 1.25 ±0.05 | 1.28 ±0.04 | 1.26 ±0.06 |
| Positive Control | 1.50 ±0.10 | 1.67 ±0.15 | 1.78 ±0.16 | 1.87 ±0.21 | 1.89 ±0.19 | 2.00 ±0.21 |
| Cryogenine | 1.27 ±0.03 | 1.31 ±0.03 | 1.55 ±0.05[a] | 1.37 ±0.06 | 1.43 ±0.03 | 1.61 ±0.04 |
| Phenylbutazone | 1.30 ±0.02[a] | 1.31 ±0.02 | 1.32 ±0.02 | 1.35 ±0.04 | 1.46 ±0.06 | 1.48 ±0.04 |
| Mefenamic Acid | 1.19 ±0.02 | 1.26 ±0.01 | 1.29 ±0.01 | 1.39 ±0.02 | 1.57 ±0.04[a] | 1.74 ±0.04[a] |
| Indomethacin | 1.34 ±0.05[a] | 1.43 ±0.05[a] | 1.45 ±0.07[a] | 1.41 ±0.09[a] | 1.57 ±0.10[a] | 1.69 ±0.10[a] |
| Paramethasone | 1.10 ±0.01 | 1.17 ±0.02 | 1.15 ±0.02 | 1.18 ±0.01 | 1.19 ±0.02 | 1.22 ±0.06 |
| Hydrocortisone | 1.26 ±0.02 | 1.29 ±0.04 | 1.31 ±0.04 | 1.44 ±0.04[a] | 1.51 ±0.04[a] | 1.56 ±0.04[a] |
| 6-mercaptopurine | 1.20 ±0.03 | 1.21 ±0.02 | 1.26 ±0.03 | 1.34 ±0.05 | 1.35 ±0.04 | 1.41 ±0.03 |

[a] Not significantly different from Positive Control group values ($P = 0.05$)

Table V.-- Effects of Drug Treatments on Non-Injected-Paw Swelling in Adjuvant-Induced Arthritis

| | Percent Inhibition of Increased Foot Volume | | | | | |
|---|---|---|---|---|---|---|
| Treatment Group | Day 15 | Day 18 | Day 21 | Day 24 | Day 27 | Day 30 |
| Negative Control | -- | -- | -- | -- | -- | -- |
| Positive Control | -- | -- | -- | -- | -- | -- |
| Cryogenine | 93.5 | 87.5 | 86.4 | 81.5 | 48.6 | 55.6 |
| Phenylbutazone | 74.2 | 81.3 | 83.1 | 80.0 | 65.7 | 67.9 |
| Mefenamic Acid | 112.9 | 93.8 | 89.8 | 75.4 | 51.4 | 37.0 |
| Indomethacin | 71.0 | 62.5 | 66.1 | 75.4 | 54.3 | 45.7 |
| Paramethasone | 125.8 | 102.0 | 105.1 | 100.0 | 98.6 | 95.1 |
| Hydrocortisone | 90.3 | 87.5 | 86.4 | 67.7 | 60.0 | 59.3 |
| 6-mercaptopurine | 103.0 | 100.0 | 91.5 | 80.0 | 80.0 | 75.3 |

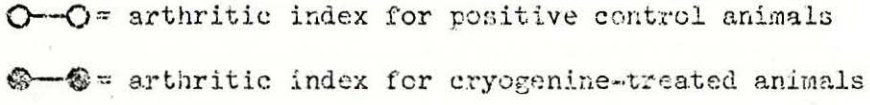
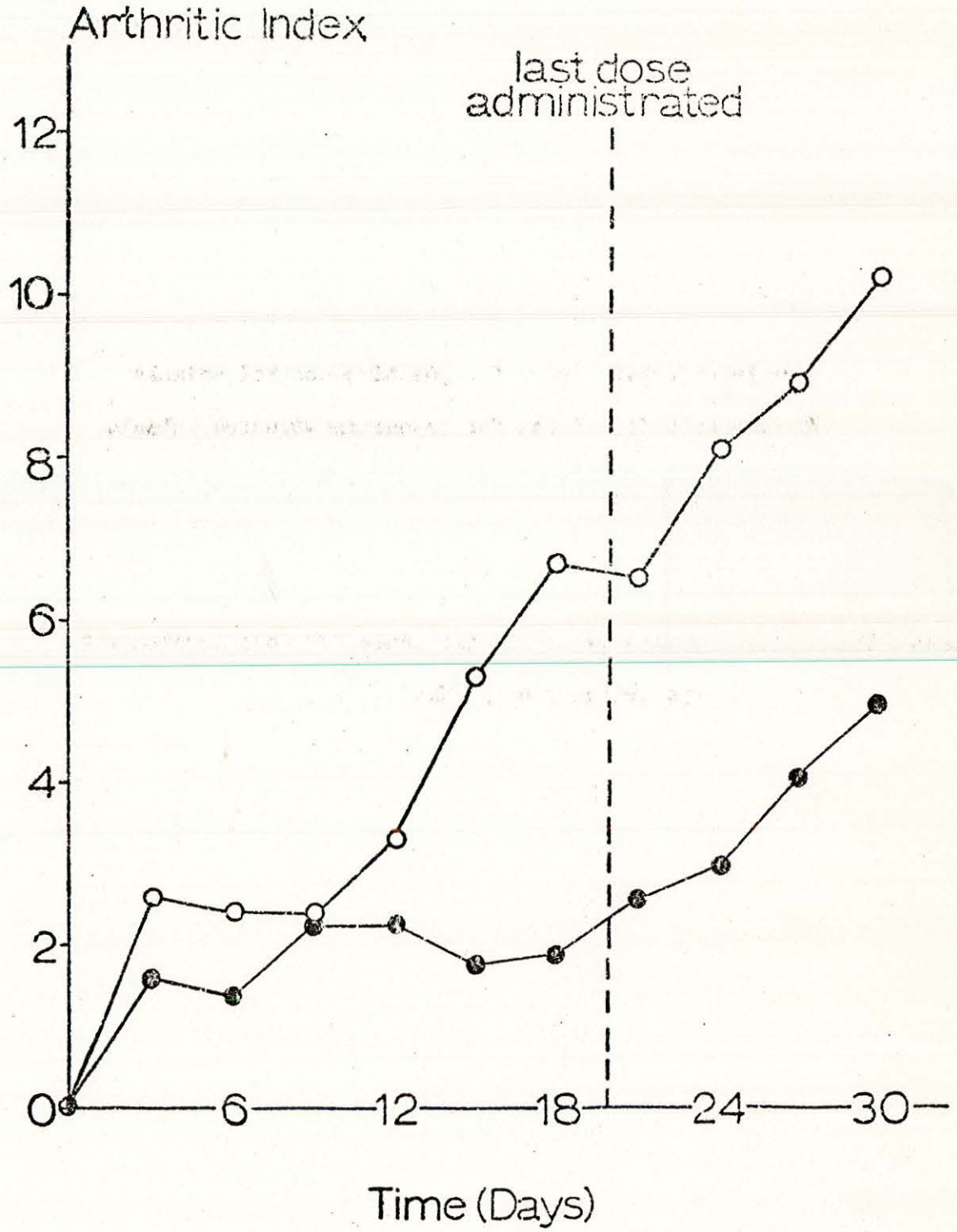



Figure 13.-- Comparative arthritic index for rats treated with cryogenine (100 mg./Kg.)


Arthritic Index
last dose
administrated
12
10
8
6
4
2
0
6
12
18
24
30
Time (Days)

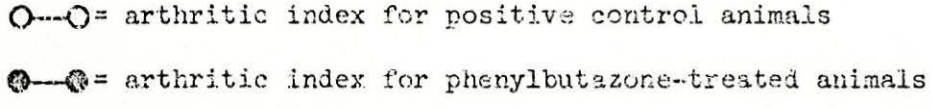
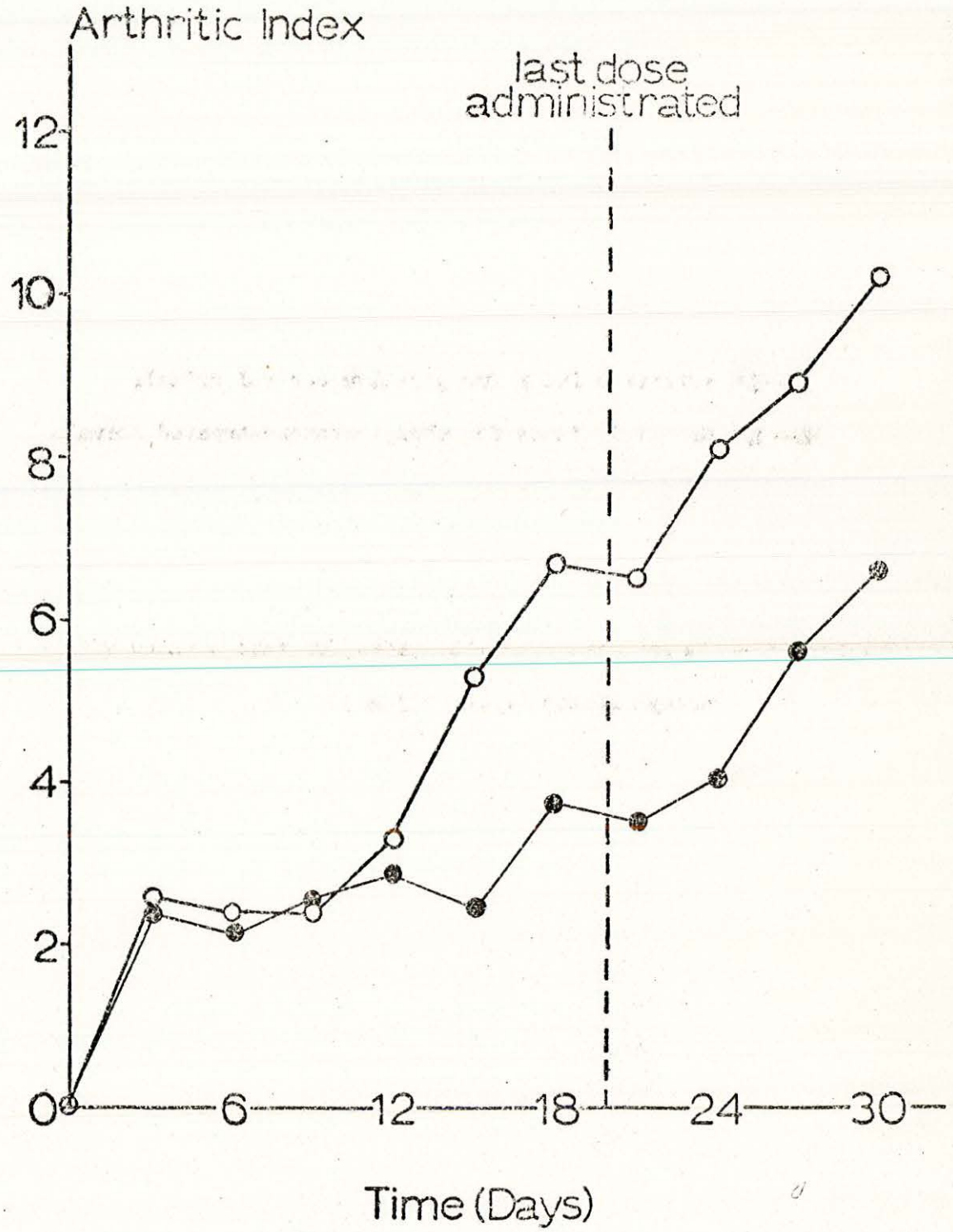



Figure 14.-- Comparative arthritic index for rats treated with phenylbutazone (100 mg./Kg.)


Arthritic Index
last dose
administrated
12
10
8
6
4
2
0
6
12
18
24
30
Time (Days)

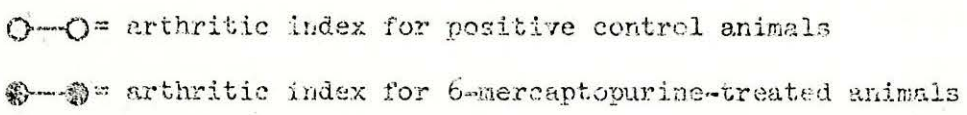
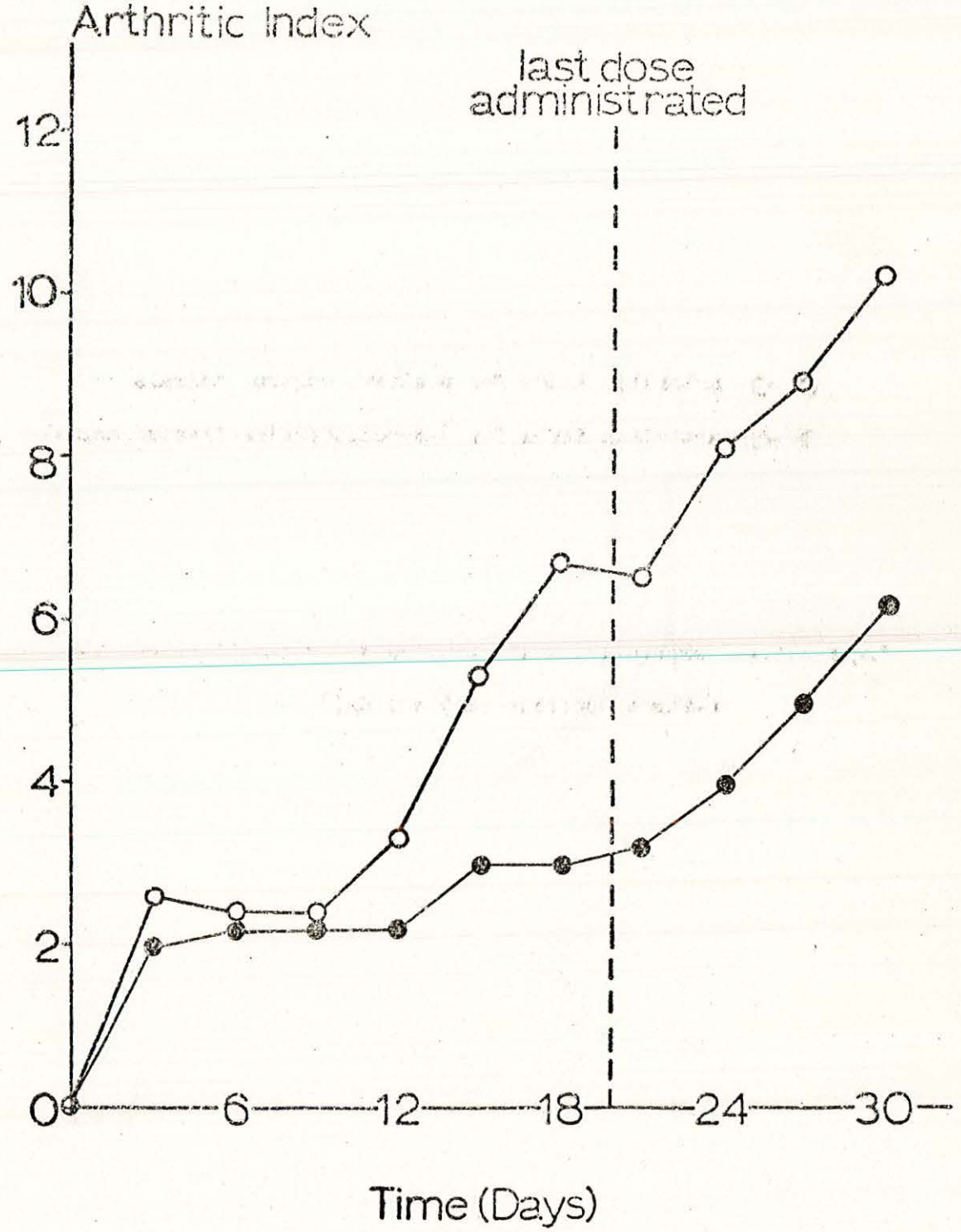



Figure 15.— Comparative arthritic index for rats treated with 6-mercaptopurine (2.0 mg./Kg.)


Arthritic Index
last dose
administrated
12
10
8
6
4
2
0
6
12
18
24
30
Time (Days)

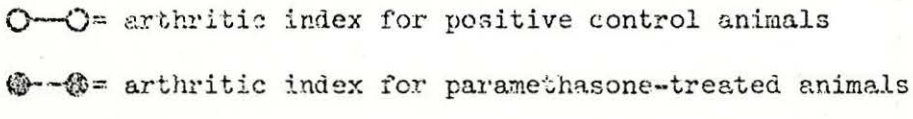
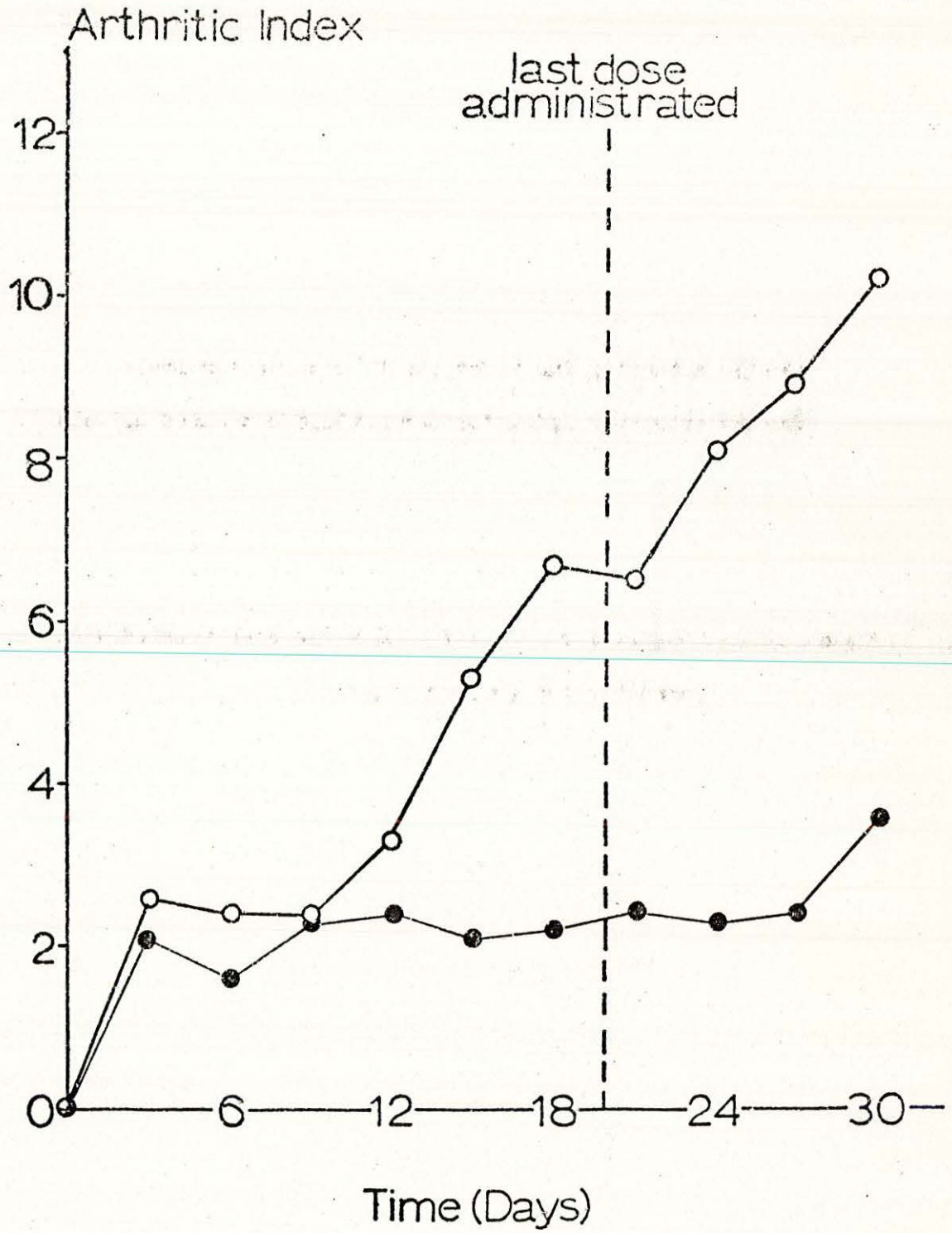



Figure 16.-- Comparative arthritic index for rats treated with paramethasone (0.5 mg./Kg.)


Arthritic Index
last dose
administrated
12
10
8
6
4
2
0
6
12
18
24
30
Time (Days)

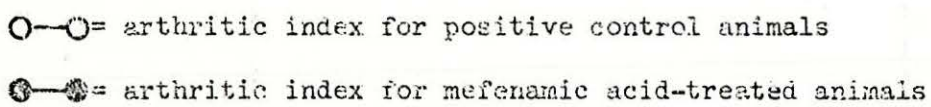
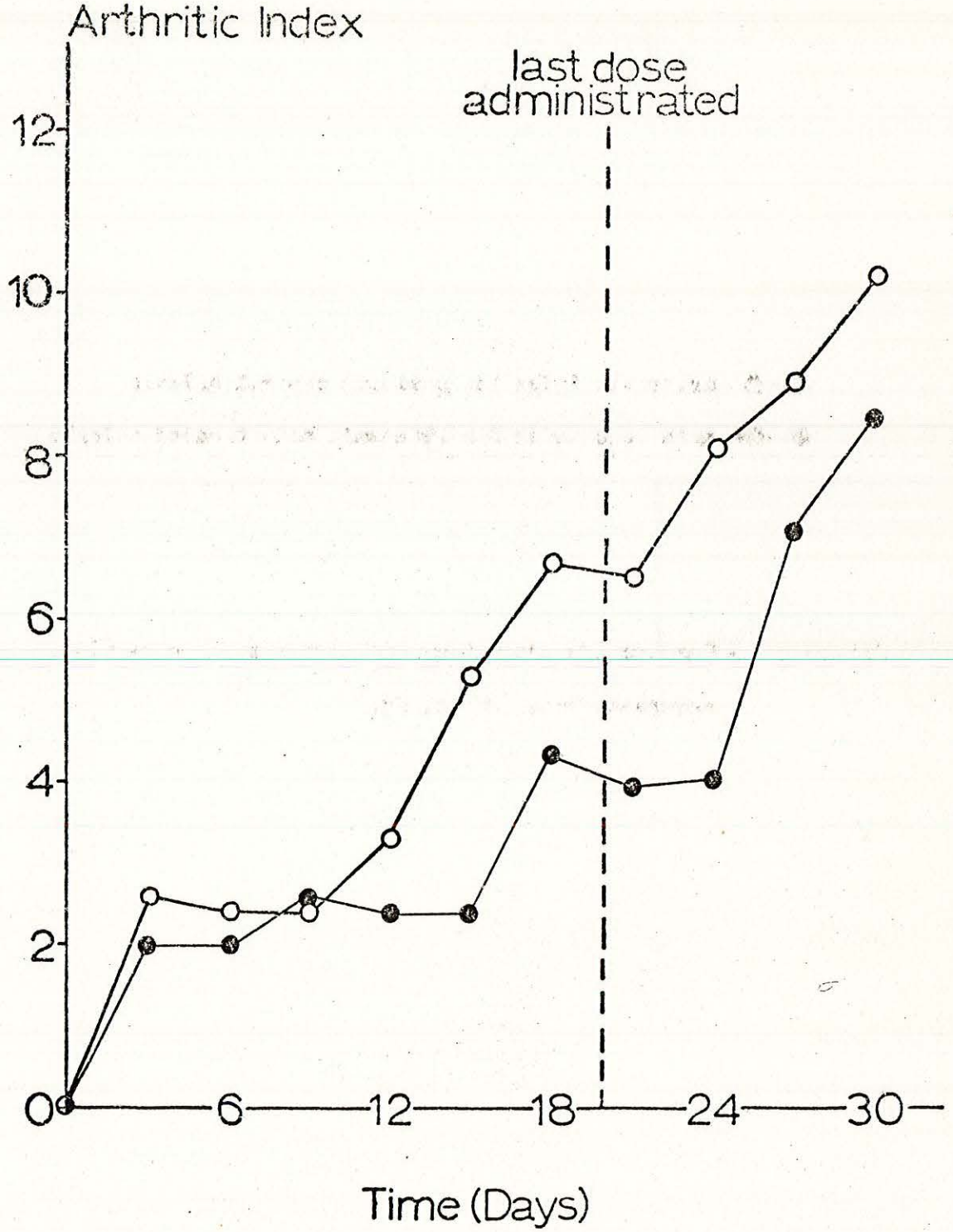



Figure 17.-- Comparative arthritic index for rats treated with mefenamic acid (25 mg./Kg.)


Arthritic Index
last dose
administrated
12
10
8
6
4
2
0
6
12
18
24
30
Time (Days)

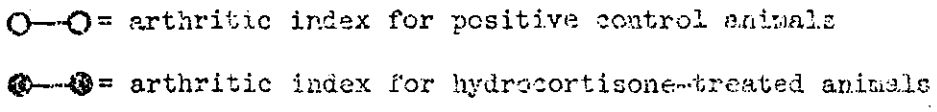
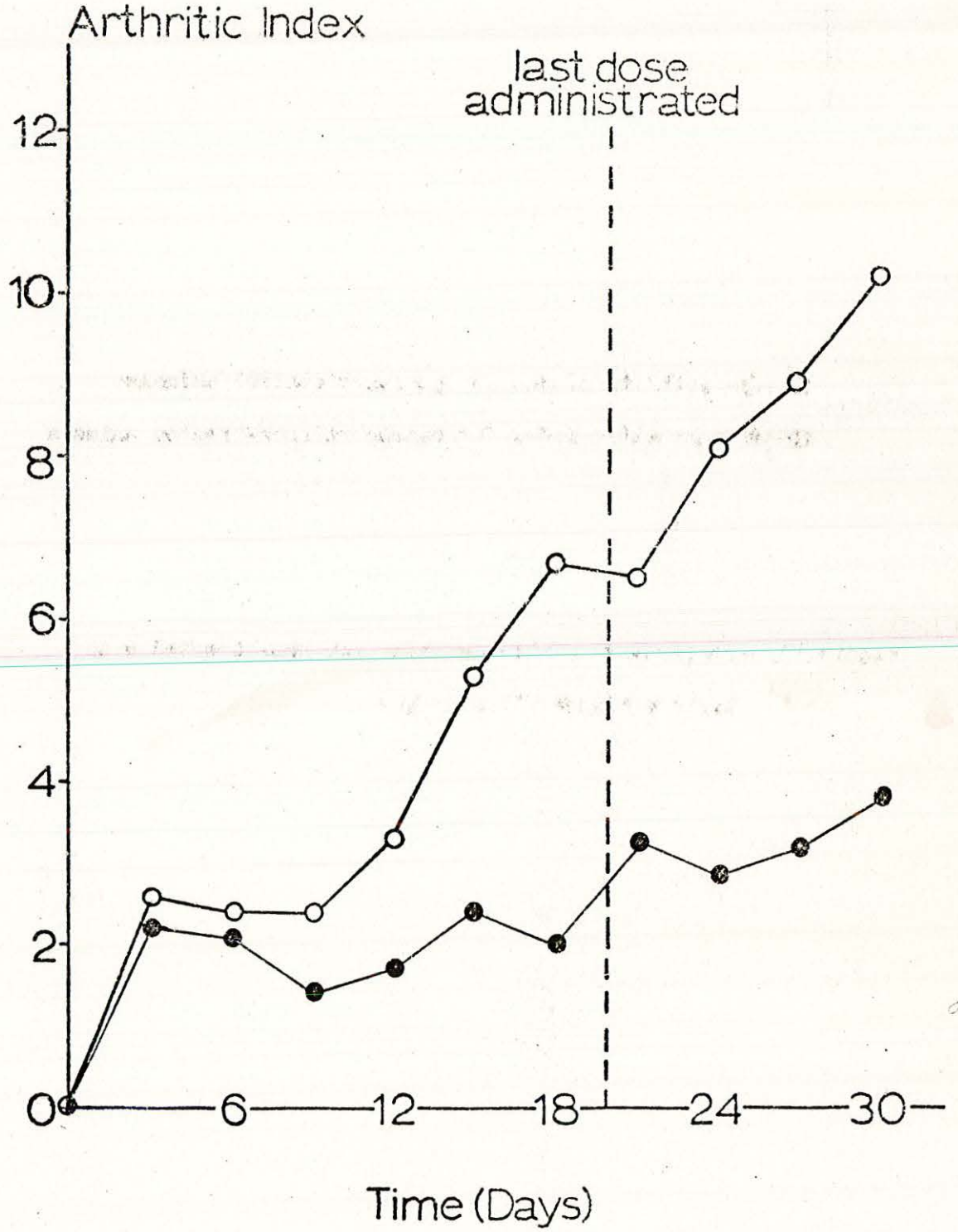



Figure 18.--Comparative arthritic index for rats treated with hydrocortisone (10 mg./Kg.)


Arthritic Index
last dose
administrated
12
10
8
6
4
2
0
6
12
18
24
30
Time (Days)

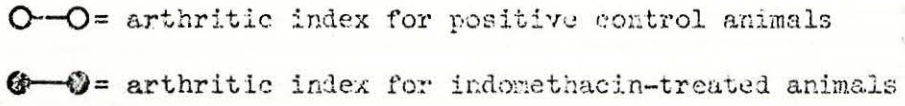
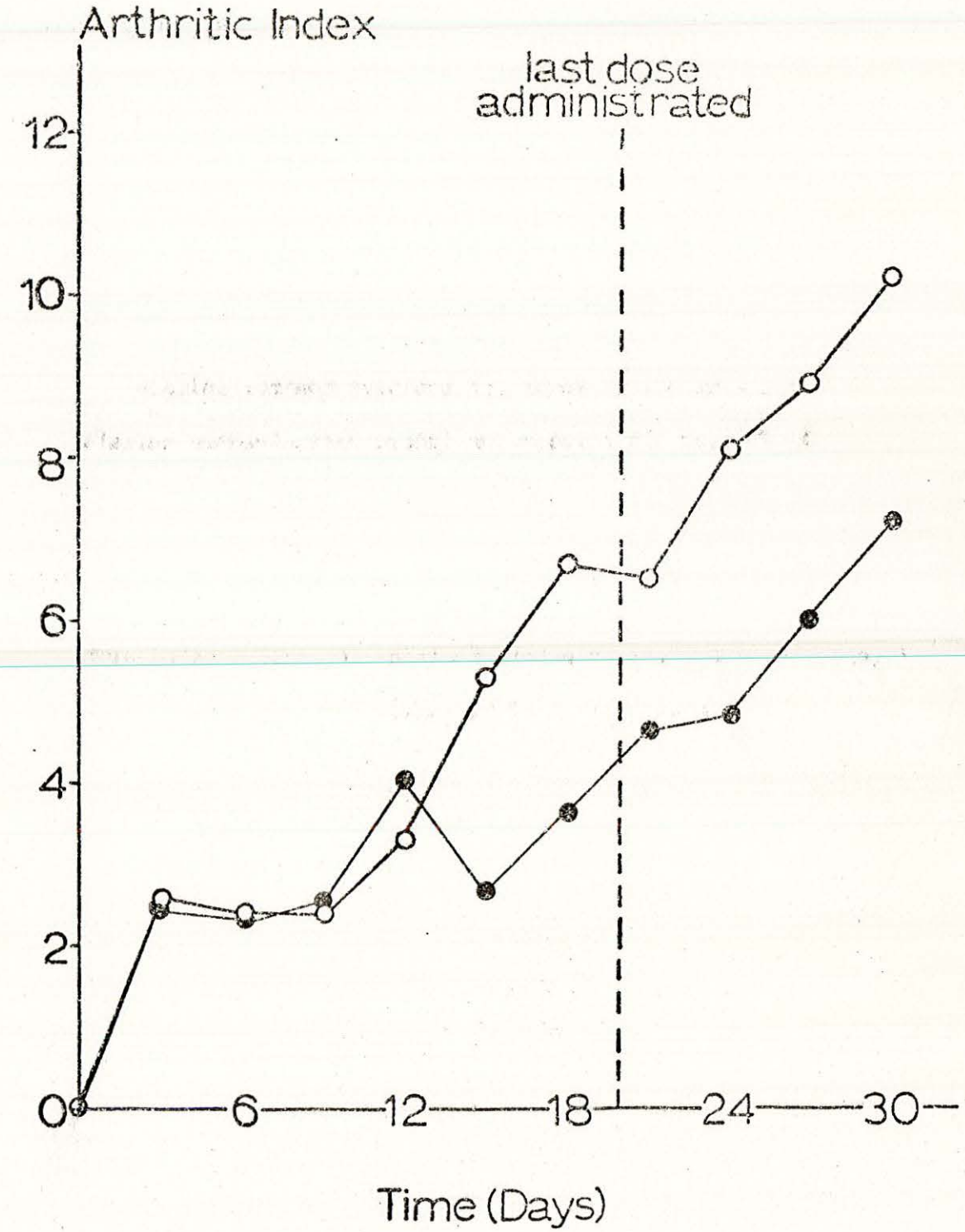



Figure 19.-- Comparative arthritic index for rats treated with indomethacin (1.0 mg./Kg.)


Arthritic Index
last dose
administrated
12
10
8
6
4
2
0
6
12
18
24
30
Time (Days)

determined by the visual scoring system. Treatment of all animals was initiated one day prior to adjuvant injection and was continued through day "20". Unless indicated otherwise, each point represents the mean arthritic index score for a group of 10 rats on a particular day.

All animals in the positive control group (receiving agar vehicle only) developed either moderate or severe polyarthritic lesions which were first observed on day "12" to day "15" and progressively increased in severity for the duration of the 30 day observation period. The cytotoxic agent, 6-mercaptopurine, and (to a lesser extent) paramethasone and hydrocortisone were the only agents tested which produced significant protection against the initial inflammatory response (day "0" through "6"). However, rats treated with cyogenine and the other selected therapeutic agents, (with the exception of indomethacin) were afforded significant protection during the acute phase of the arthritic disease. The arthritic index and therapeutic efficacy for all treatments are summarized in Table VI. Following termination of drug treatment (day "20") the severity of the arthritic syndrome increased in all groups; however, significant protection was still evident at the end of the 30 day study in rats treated with cryogenine, hydrocortisone, 6-mercaptopurine and phenylbutazone. The various manifestations of the disseminated arthritic disease did not appear in the

**Table VI.-- Arthritic Index and Therapeutic Efficacy of Drug Treatments in Adjuvant-induced Arthritis**

| | Arthritic Index | | | Percent Inhibition[a] | | |
|---|---|---|---|---|---|---|
| Treatment Group | Day 12 | Day 21 | Day 30 | Day 12 | Day 21 | Day 30 |
| Positive Control | 3.3 | 6.5 | 10.2 | -- | -- | -- |
| Cryogenine | 2.0[b] | 3.2[b] | 6.2[b] | 39.4 | 50.8 | 39.2 |
| Phenylbutazone | 2.9 | 3.6[b] | 6.7[b] | 12.1 | 44.6 | 34.3 |
| Mefenamic Acid | 2.4 | 4.0[b] | 8.6 | 27.3 | 38.5 | 15.7 |
| Indomethacin | 3.1 | 4.7 | 7.3 | 6.1 | 27.7 | 28.4 |
| Paramethasone | 2.4 | 2.4[b] | 3.6[b] | 27.3 | 63.1 | 64.7 |
| Hydrocortisone | 1.7[b] | 3.3[b] | 3.9[b] | 48.5 | 49.2 | 61.8 |
| 6-mercaptopurine | 2.3[b] | 2.6[b] | 4.9[b] | 30.3 | 60.0 | 50.9 |

[a] Determined by comparison of drug-treated groups with positive control group values

[b] Significantly different from Positive Control group values (P = 0.05)

paramethasone treated group for 8 days after drug treatment was terminated.

Impairment of Body Growth.--- Body weights were recorded at the start of drug treatment (day "0") and at 3 day intervals to the conclusion of the observation period (day "30"). There were marked differences in body weight patterns between nonarthritic and arthritic control group animals (Table VII. ) In rats injected with the Mycobacterium adjuvant, the average gain in body weight was only about 5 grams during the observation period, in contrast to the negative (noninjected) control animals which gained 41 grams during the same period of time. Arthritic rats showed a very significant loss of weight during the first three days following adjuvant injection, after which a slow "recovery" to preinjection weights occurred by day "12". Body weight then fluctuated over a narrow range during the remaining period of study. In this respect, the results obtained are similar to those of Glenn et al. (35) and intermediate between those of Pearson (36) who described normal weight gain and Newbould (27) who noted severe weight loss in positive control group animals.

The body growth curves for drug-treated arthritic rats (Figure 20. to Figure 26.) indicate that among the nonsteroidal antiinflammatory agents studied, phenylbutazone, mefenamic acid and indomethacin (at the dose levels employed) did not significantly alter body weight relative

Table VII.--Effects of Drug Treatments on Body Weight Gain in Adjuvant-Induced Arthritis

| Treatment Group | Mean Body Weight (gm.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 3 | Day 6 | Day 12 | Day 18 | Day 21 | Day 24 | Day 30 |
| Negative Control | 228 | 232[a] | 234[a] | 235[a] | 253[a] | 253[a] | 258[a] | 269[a] |
| Positive Control | 215 | 204 | 209 | 217 | 213 | 216 | 212 | 220 |
| Cryogenine | 220 | 215[a] | 217 | 222 | 229 | 234[a] | 235[a] | 241[a] |
| Phenylbutazone | 212 | 210 | 216 | 221 | 218 | 222 | 224 | 231 |
| Mefenamic Acid | 217 | 212 | 215 | 223 | 227 | 226 | 229 | 236 |
| Indomethacin | 216 | 206 | 205 | 214 | 216 | 219 | 224 | 230 |
| Paramethasone | 210 | 190[a] | 186[a] | 183[a] | 191[a] | 190[a] | 194 | 202 |
| Hydrocortisone | 226 | 219[a] | 218 | 221 | 228 | 230 | 236[a] | 244[a] |
| 6-mercaptopurine | 218 | 205 | 198 | 198[a] | 201 | 199 | 200 | 208 |

[a] Significantly different from Positive Control group values ($P = 0.05$)

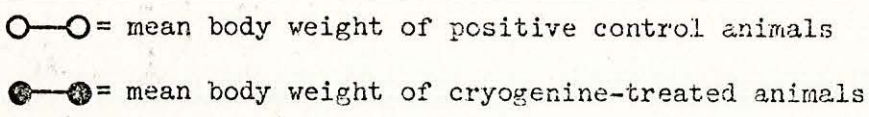
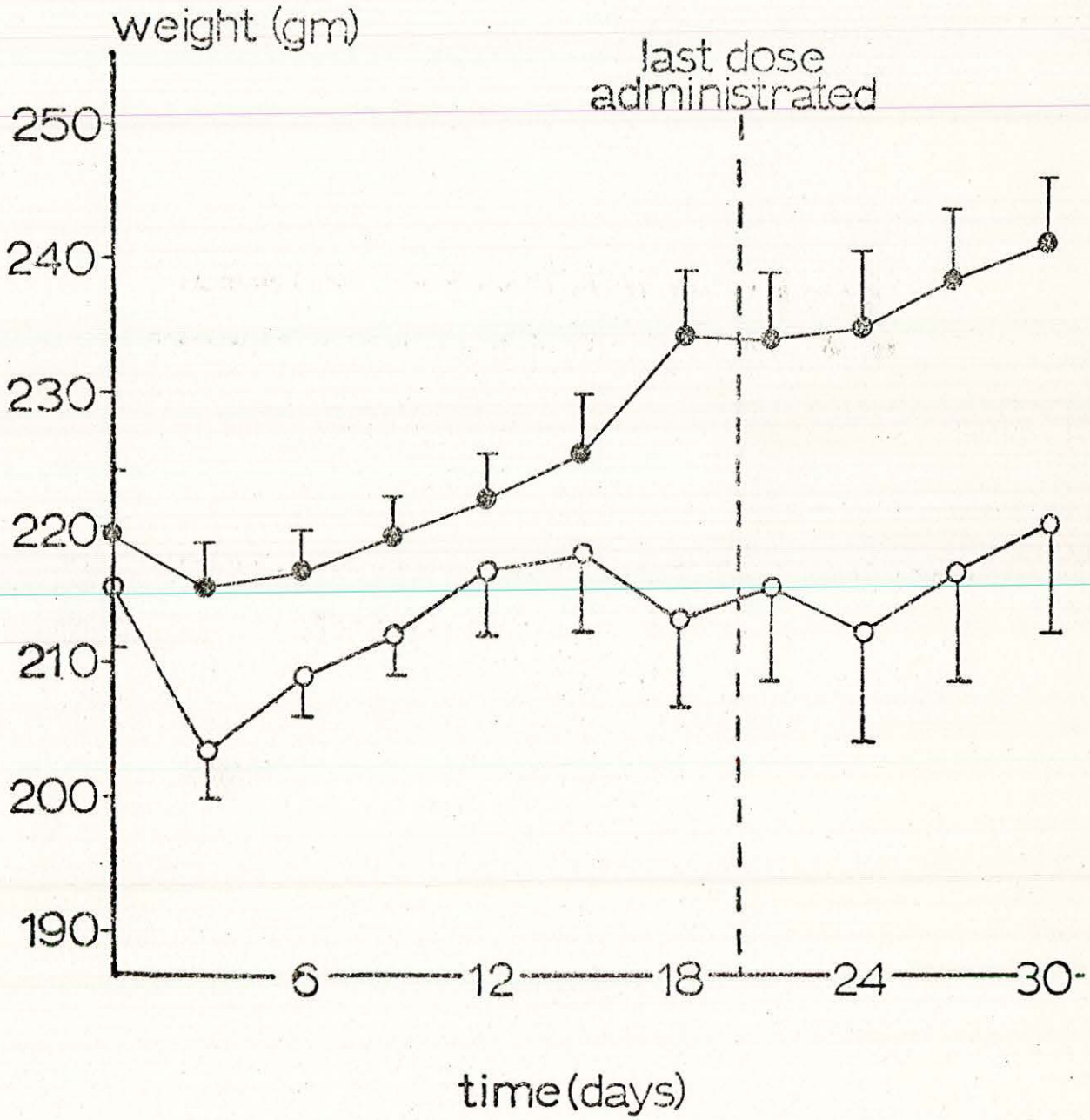


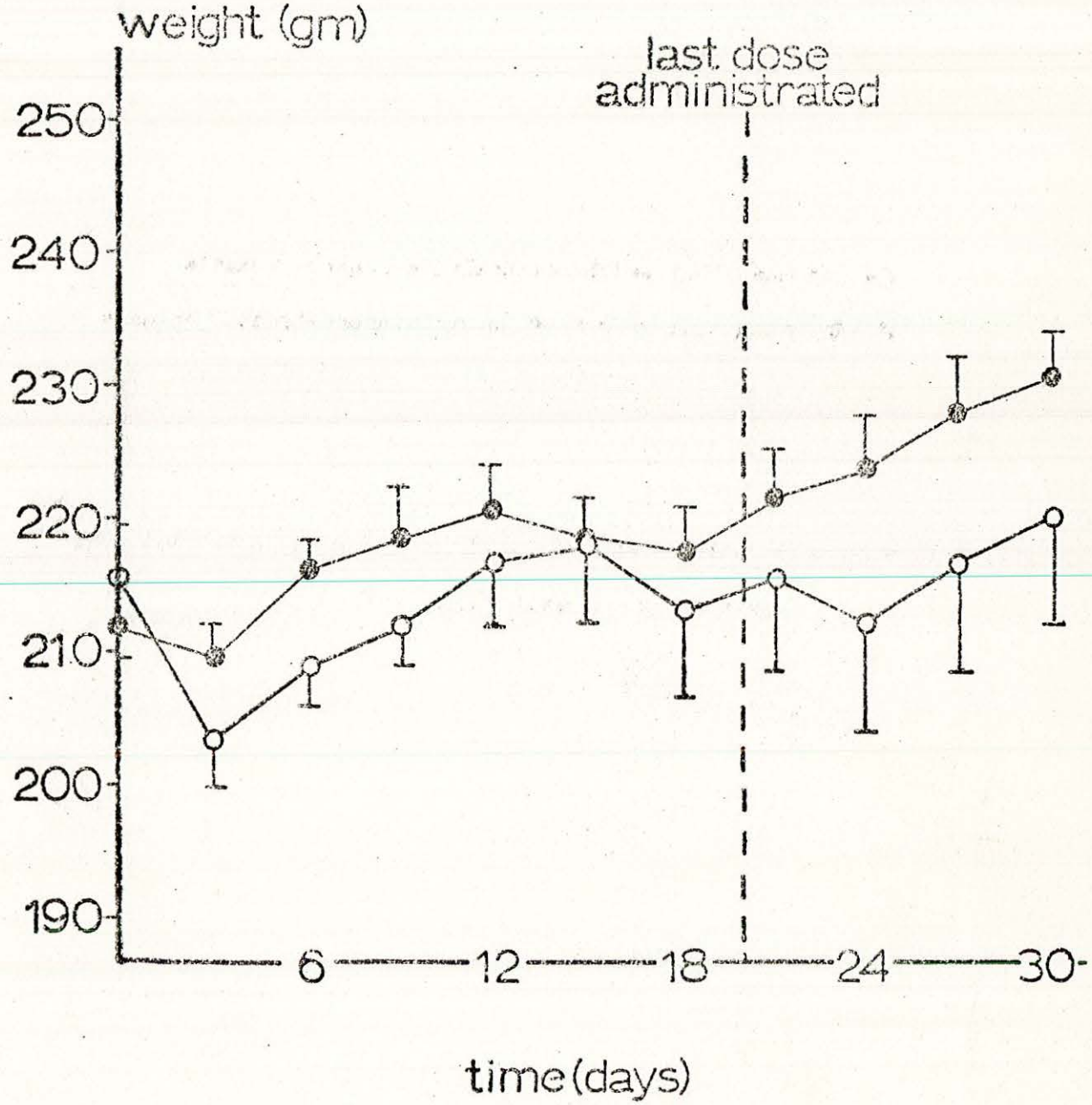

Figure 20.-- Growth impairment in adjuvant-induced arthritic rats treated with cryogenine (100 mg./Kg.)


weight (gm)
last dose
administrated
250
240
230
220
210
200
190
6
12
18
24
30
time (days)

O—O = mean body weight of positive control animals

●—● = mean body weight of phenylbutazone-treated animals

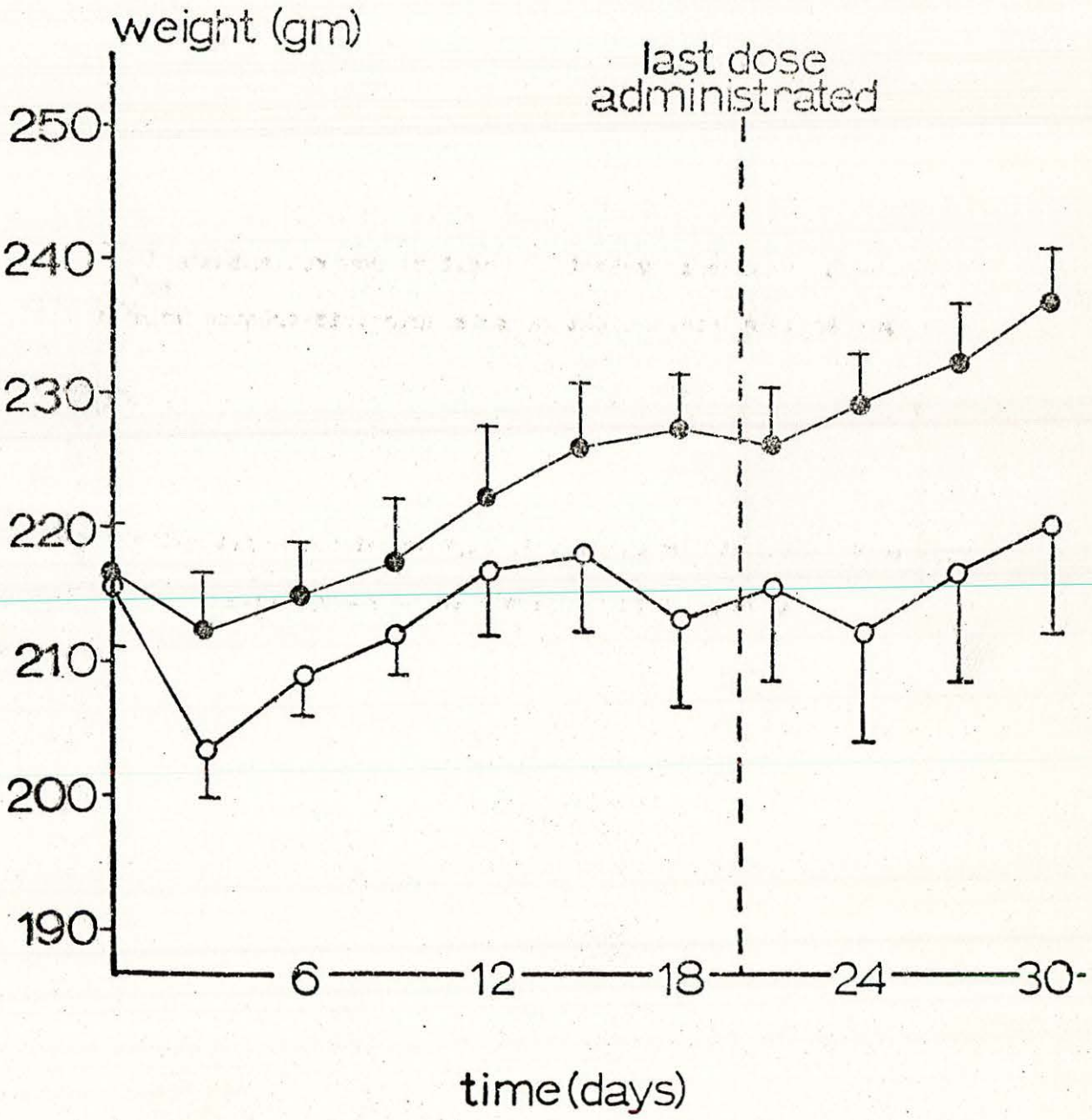
Figure 21.-- Growth impairment in adjuvant-induced arthritic rats treated with phenylbutazone (100 mg./Kg.)


weight (gm)
last dose
administrated
250
240
230
220
210
200
190
6
12
18
24
30
time (days)

O—O = mean body weight of positive control animals

●—● = mean body weight of mefenamic acid-treated animals

Figure 22.-- Growth impairment in adjuvant-induced arthritic rats treated with mefenamic acid (25 mg./Kg.)


weight (gm)
last dose
administrated
250
240
230
220
210
200
190
6
12
18
24
30
time (days)

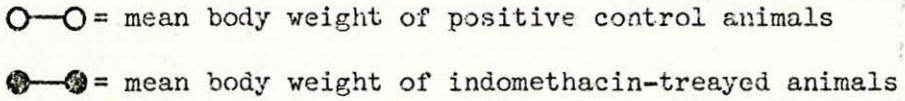
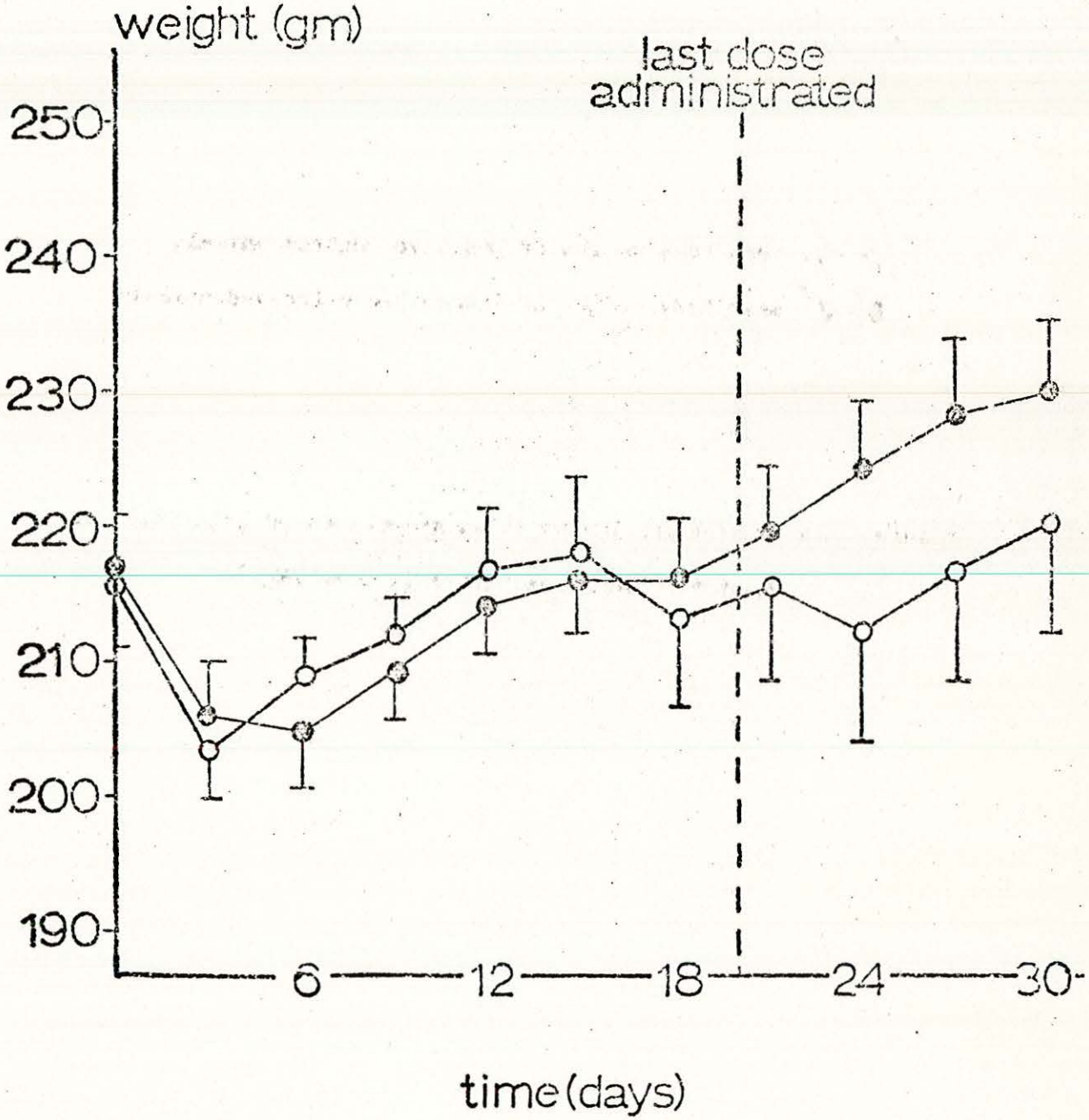



Figure 23.-- Growth impairment in adjuvant-induced arthritic rats treated with indomethacin (1.0 mg./Kg.)


weight (gm)
last dose
administrated
250
240
230
220
210
200
190
6
12
18
24
30
time (days)

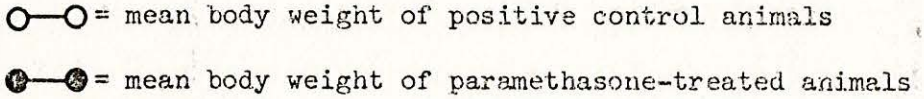
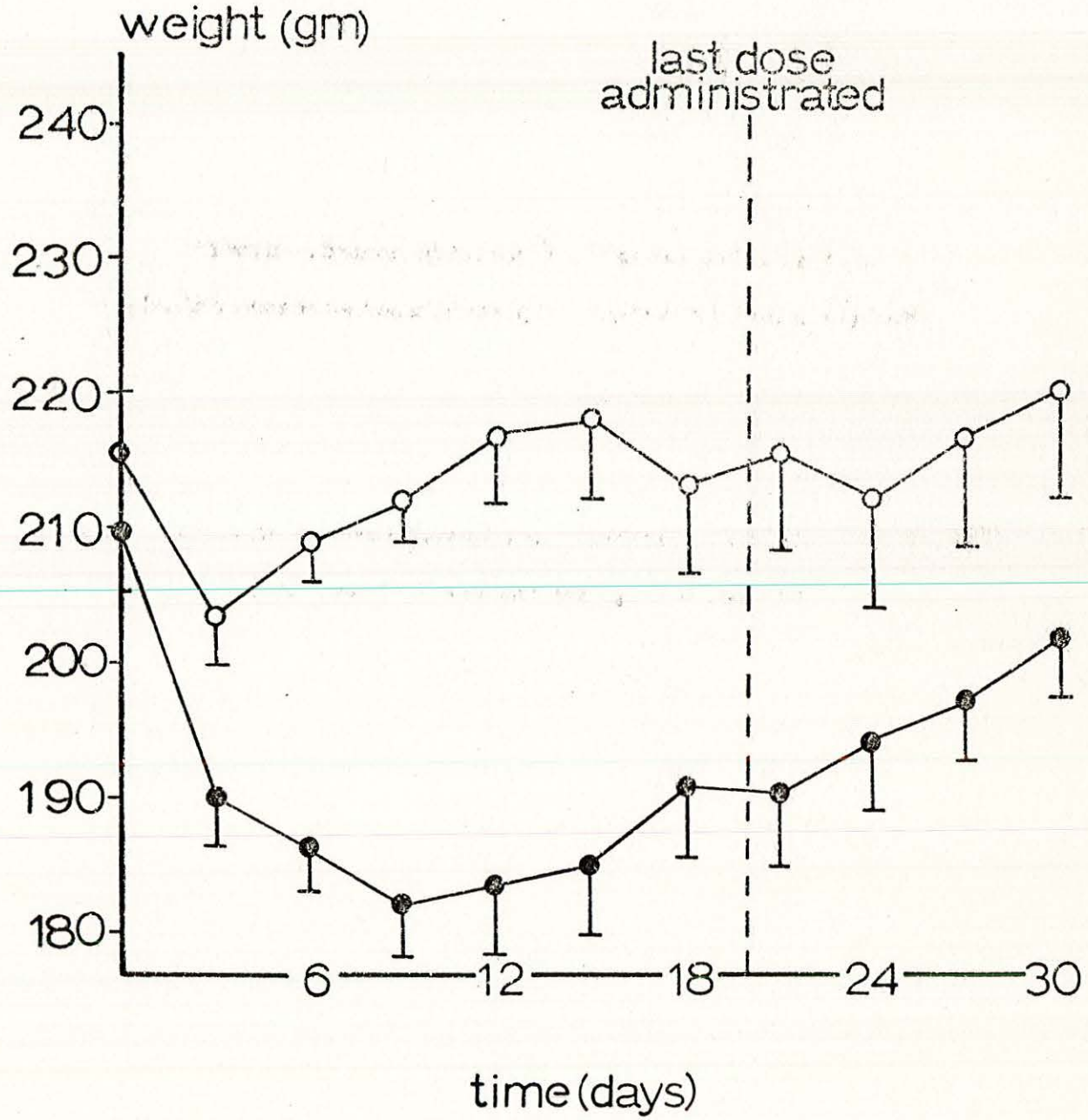



Figure 24.-- Growth impairment in adjuvant-induced arthritic rats treated with paramethasone (0.5 mg./Kg.)


weight (gm)
last dose
administrated
240
230
220
210
200
190
180
6
12
18
24
30
time (days)

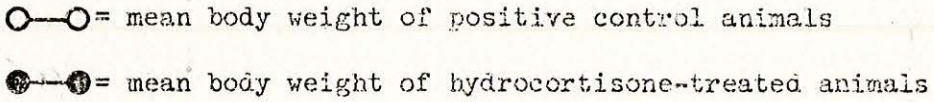
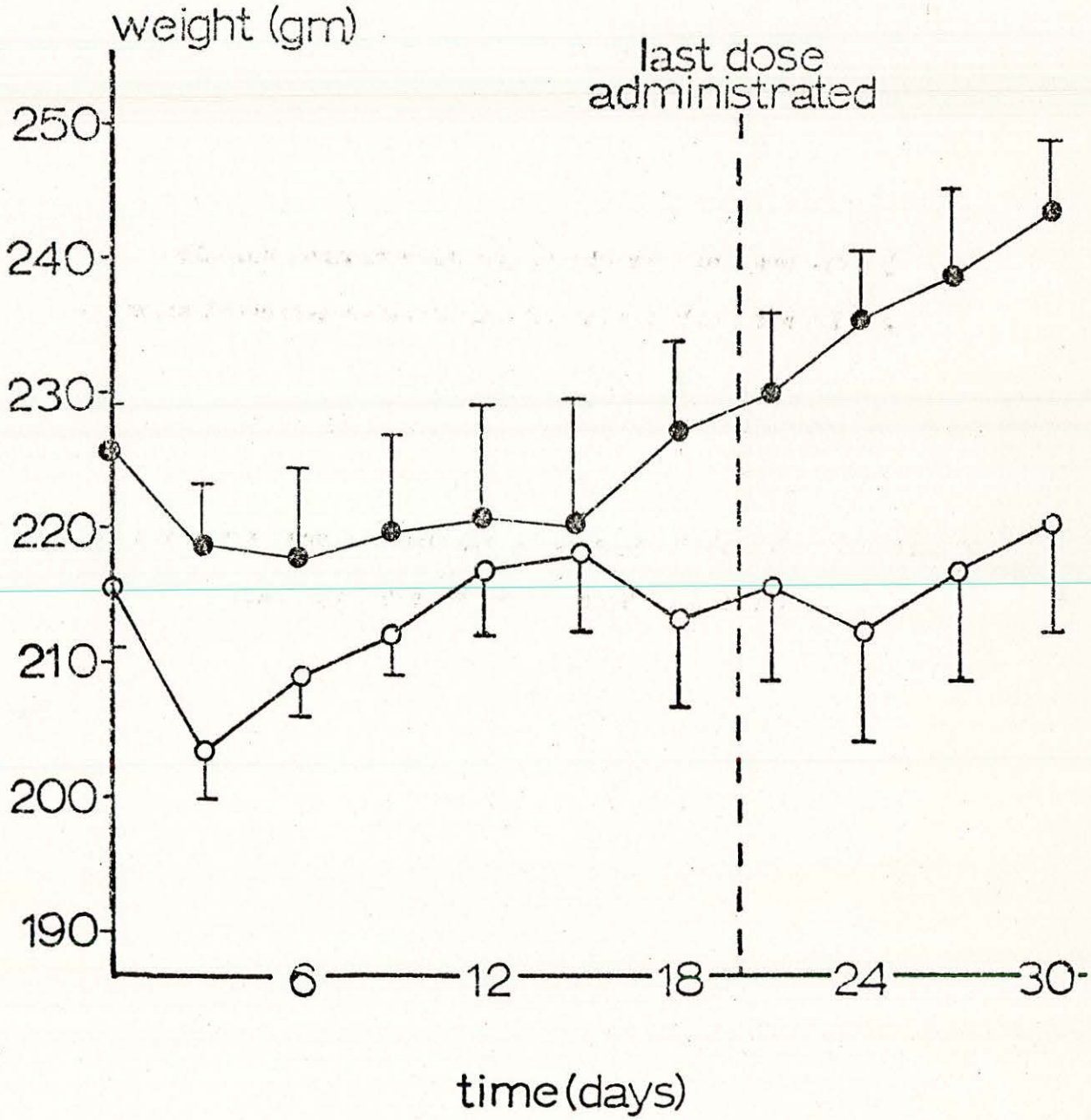


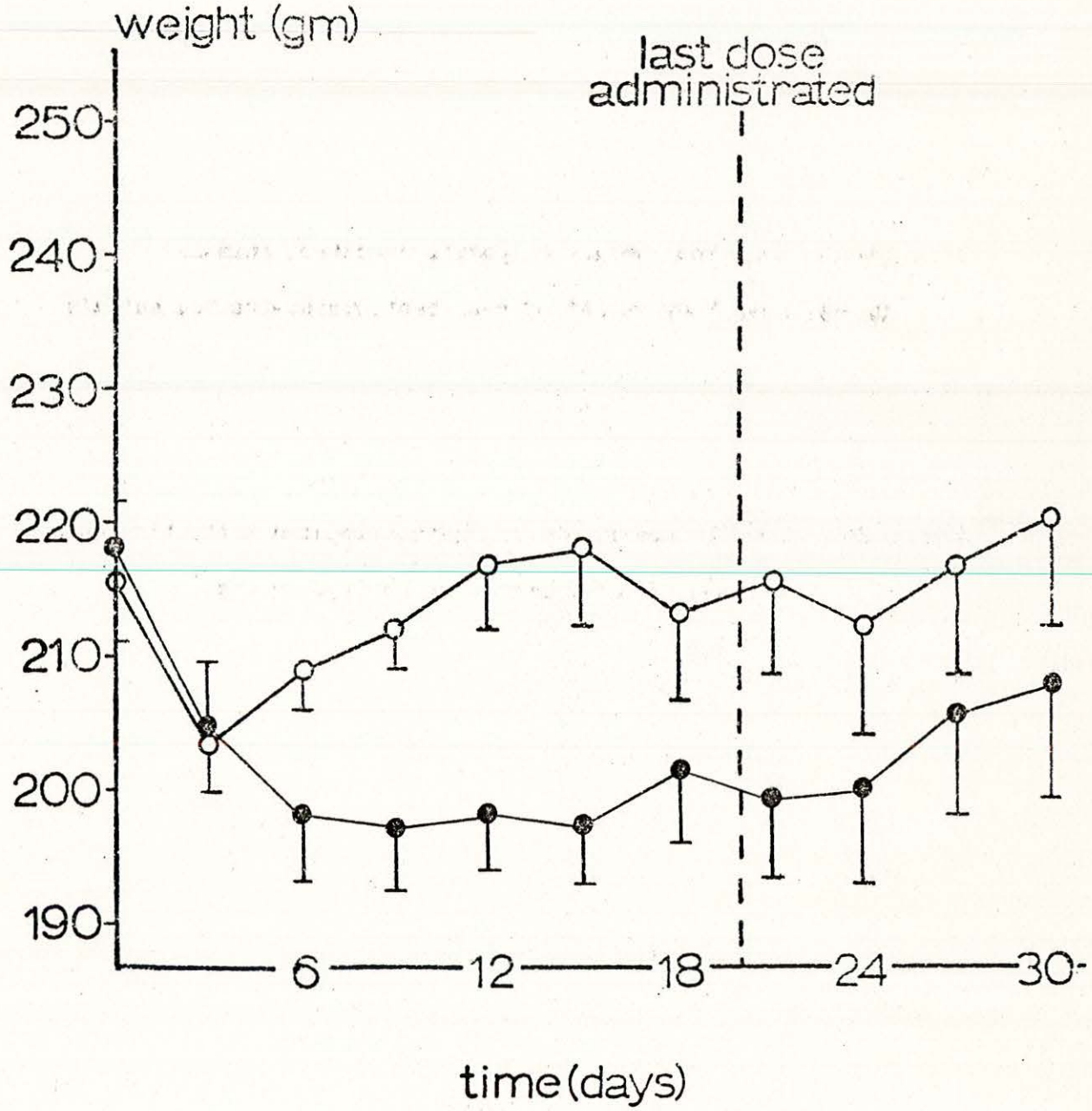

Figure 25.-- Growth impairment in adjuvant-induced arthritic rats treated with hydrocortisone (10 mg./Kg.)


weight (gm)
last dose
administrated
250
240
230
220
210
200
190
6
12
18
24
30
time (days)

O---O = mean body weight of positive control animals

●—● = mean body weight of 6-mercaptopurine-treated animals

Figure 26.-- Growth impairment in adjuvant-induced arthritic rats treated with 6-mercaptopurine (2.0 mg./Kg.)


weight (gm)
last dose
administrated
250
240
230
220
210
200
190
6
12
18
24
30
time (days)

to positive control animals. Cryogenine actually promoted an increase in body weight after termination of treatment (day "21" to day "30"); this gain was significantly different (P 0.05) from that of nontreated adjuvant-injected controls. In arthritic rats, 6-mercaptopurine and paramethasone caused weight loss while the body weight of hydrocortisone-treated animals was significantly greater than positive control animals in the termination of the observation period (day "24" through day "30").

The therapeutic action of cryogenine and the selected antiinflammatory agents on adrenal, spleen and thymus weights are summarized in Table VIII. By the end of the 15 day period, the inflammatory process had developed to a sufficient intensity to markedly increase adrenal and splenic weights and to decrease thymus weights in positive control group animals. These changes still persisted at the 21 day period. At the conclusion of the 30 day observation period, however, the increase in adrenal weight was no longer so apparent, however, the decrease in thymus weights was observed to persist.

Effects of Cryogenine on Previously Established Arthritis.— Arthritis was induced in a number of rats by methods described previously and allowed to proceed without drug treatment until day "21", at which time the animals were divided into two groups. One group received only the 0.25% agar orally while the other was treated with cryogenine (100 mg/Kg, p.o.) daily for 7 days. The

Table VIII.

Comparative Effects in The Rat of Selected Drugs on Wet Organ Weights in The Presence of Adjuvant-Induced Arthritis.

Mean Organ Weights (mg./100 gm. Body Weight) ±S.E.

| Treatment Group | Day 15 | | | Day 21 | | | Day 30 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Spleen | Adrenals | Thymus | Spleen | Adrenals | Thymus | Spleen | Adrenals | Thymus |
| Negative Control | 247 ±19 | 17.4 ±1.2 | 181 ± 2 | 254 ±10 | 16.3 ±0.6 | 185 ±11 | 283 ± 9 | 17.2 ±0.5 | 220 ±18 |
| Positive Control | 386 ±14 | 20.9 ±1.0 | 119 ±13 | 484 ±32 | 23.6 ±1.8 | 122 ±14 | 369 ±22 | 17.8 ±2.2 | 96 ± 8 |
| Cryogenine | 306 ±12 | 16.8 ±0.9 | 175 ±19 | 364 ±28 | 18.2 ±1.8 | 199 ±21 | 304 ±10 | 16.3 ±1.2 | 188 ±10 |
| Phenylbutazone | 321 ±22 | 17.9 ±1.7 | 138 ±28 | 341 ±22 | 19.6 ±1.2 | 102 ±18 | 318 ±28 | 17.9 ±1.6 | 136 ±16 |
| Indomethacin | 370 ±54 | 22.1 ±0.7 | 146 ±17 | 399 ±27 | 21.0 ±1.4 | 182 ± 9 | 318 ±63 | 17.7 ±2.2 | 176 ± 2 |
| Mefenamic Acid | 369 ±36 | 20.0 ±2.4 | 118 ±19 | 460 ±46 | 21.8 ±1.0 | 90 ±17 | 400 ±32 | 18.2 ±0.1 | 100 ±14 |
| Paramethasone | 176 ±18 | 10.0 ±1.1 | 68 ±11 | 223 ±18 | 11.2 ±2.1 | 130 ± 8 | 270 ±66 | 14.6 ±0.8 | 147 ±22 |
| Hydrocortisone | 240 ±28 | 12.6 ±0.7 | 71 ±13 | 250 ±18 | 12.5 ±1.8 | 140 ±29 | 286 ±22 | 17.2 ±1.4 | 113 ±11 |
| 6-Mercaptopurine | 226 ±27 | 17.0 ±0.8 | 65 ± 3 | 264 ±17 | 18.2 ±0.8 | 78 ± 6 | 332 ±32 | 18.4 ±0.6 | 98 ± 9 |

results of this study are shown in Table IX. Cryogenine, when administered to rats with the established disease, exerted a significant inhibitory effect (paw volume) within 4 days following initiation of drug treatment. Moreover, animals receiving cryogenine showed a greater body weight gain during the treatment period than did the arthritic control group; however, this was not statistically significant. ($P>0.05$).

Serum Turbidity in Adjuvant-Injected Rats.--

Figure 27. summarizes the effects produced by the selected antiinflammatory drugs on serum coagulation in adjuvant-injected rats as reflected by comparative absorbancy determinations (turbidity). Significantly decreased turbidity values were noted in serum obtained from adjuvant-injected control group animals in comparison to vehicle treated (0.25% agar) non-injected animals. Cryogenine, as well as most of the other selected non-steroidal antiinflammatory agents all tended to reverse this decrease in turbidity towards normal control levels. Hydrocortisone and paramethasone treatment likewise reversed the decrease produced by adjuvant injection. Mefenamic acid and 6-mercaptopurine, at the dose levels employed were the least effective agents in preventing the lowered turbidity values seen in adjuvant-injected animals.

Hematological Studies in Adjuvant-Injected Rats.--

Tables X. and XI. summarize the total and differential

Table IX.-- Effects of cryogenine on established adjuvant-induced arthritis[a]

| Day | Foot Volume, ml. ±S.E. | | | | Body Weight, gm. ±S.E. | |
|---|---|---|---|---|---|---|
| | Vehicle Controls | | Cryogenine Treated | | Vehicle Controls | Cryogenine Treated |
| | L | R | L | R | | |
| 21 | 1.76 ±0.12 | 2.58 ±0.03 | 1.77 ±0.96 | 2.59 ±0.44 | 220 ±5.48 | 221 ±2.55 |
| 22 | 1.81 ±0.13 | 2.67 ±0.03 | 1.74 ±0.13 | 2.58 ±0.19 | 221 ±6.10 | 222 ±2.78 |
| 23 | 1.79 ±0.10 | 2.73 ±0.04 | 1.80 ±0.13 | 2.47 ±0.03[b] | 224 ±5.87 | 226 ±3.19 |
| 24 | 1.92 ±0.11 | 2.86 ±0.07 | 1.66 ±0.11 | 2.35 ±0.01[b] | 218 ±3.66 | 228 ±4.13 |
| 25 | 1.96 ±0.09 | 2.91 ±0.11 | 1.64 ±0.10 | 2.29 ±0.04[b] | 218 ±3.56 | 230 ±3.97 |
| 26 | 1.97 ±0.10 | 2.88 ±0.08 | 1.66 ±0.07[b] | 2.19 ±0.04[b] | 221 ±2.56 | 230 ±3.17 |
| 27 | 1.98 ±0.09 | 2.97 ±0.14 | 1.59 ±0.07[b] | 2.15 ±0.08[b] | 219 ±1.87 | 230 ±3.37 |
| 28 | 2.05 ±0.10 | 3.08 ±0.13 | 1.65 ±0.04[b] | 2.10 ±0.09[b] | 221 ±2.17 | 229 ±3.35 |
| Net Change | +16.5% | +19.4% | -6.8% | -18.9% | +0.8 gm. | +8.3 gm. |

[a] No treatment for the first 20 days, followed by cryogenine (100 mg./Kg./day) on days 21 through 27

[b] Significantly different from vehicle controls (P = 0.05)

| Treatment Group | Oral Dosage, mg./Kg./Day |
|---|---|
| A = Negative Control | --- |
| B = Positive Control | --- |
| C = Paramethasone | 0.5 |
| D = Phenylbutazone | 100 |
| E = Mefenamic Acid | 25 |
| F = Indomethacin | 1.0 |
| G = Cryogenine | 100 |
| H = Hydrocortisone | 10 |
| I = 6-mercaptopurine | 2 |

Figure 27.-- Effects of drug treatments on serum turbidity (absorbance, mean ±S.E.) in adjuvant-induced arthritic rats. Drugs were given orally for 21 days beginning one day prior to adjuvant administration.

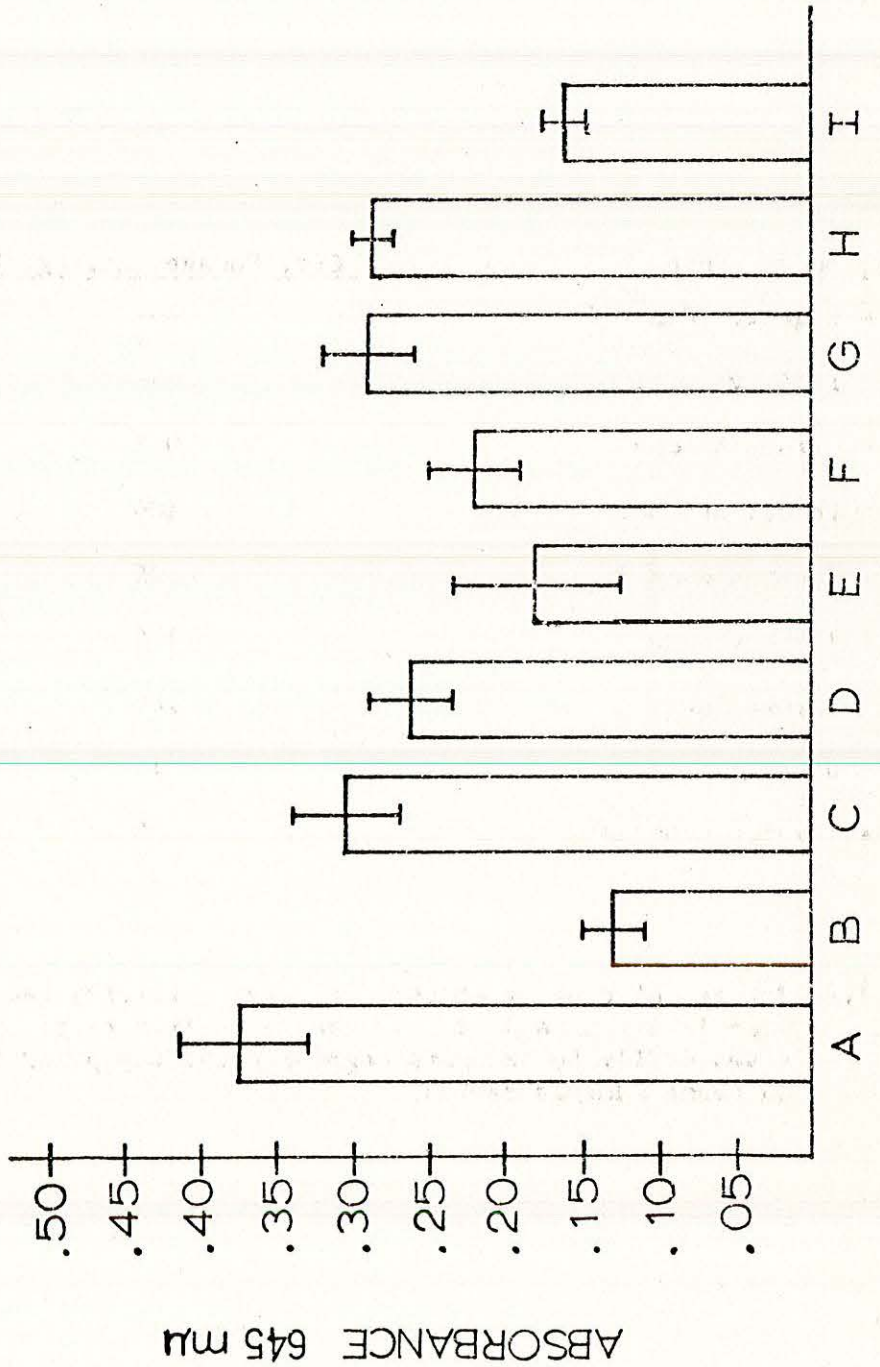

ABSORBANCE 645 mμ
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
A
B
C
D
E
F
G
H
I

Table X .-- Effects of Drug Treatments on White Cell Counts in Adjuvant-Induced Arthritis

| | Mean W.B.C. (thousands/ cu. mm.) ±S.E. | | | |
|---|---|---|---|---|
| Treatment Group | Day 6 | Day 15 | Day 21 | Day 30 |
| Negative Control | 19.3 ±0.8 | 15.4 ±0.9 | 17.3 ±1.1 | 15.3 ±0.9 |
| Positive Control | 24.7 ±2.1 | 25.2 ±3.0 | 29.2 ±1.1 | 24.5 ±3.2 |
| Cryogenine | 16.6 ±3.4 | 18.3 ±1.9 | 22.0 ±3.3 | 20.6 ±1.7 |
| Phenylbutazone | 21.4 ±0.9 | 22.2 ±3.0 | 24.4 ±2.0 | 23.9 ±2.1 |
| Mefenamic Acid | 21.4 ±1.8 | 25.3 ±0.9 | 29.3 ±3.1 | 26.3 ±2.0 |
| Indomethacin | 23.3 ±3.9 | 23.0 ±3.3 | 26.4 ±1.1 | 26.1 ±2.3 |
| Paramethasone | 21.8 ±2.2 | 10.1 ±2.3 | 13.4 ±0.3 | 18.3 ±1.8 |
| Hydrocortisone | 12.5 ±1.1 | 12.3 ±0.3 | 16.5 ±1.9 | 19.5 ±1.4 |
| 6-mercaptopurine | 14.2 ±1.9 | 24.2 ±1.1 | 26.6 ±2.2 | 25.0 ±2.7 |

Table XI.-- Effects of Drug Treatments on White Cell Counts in Adjuvant-Induced Arthritis

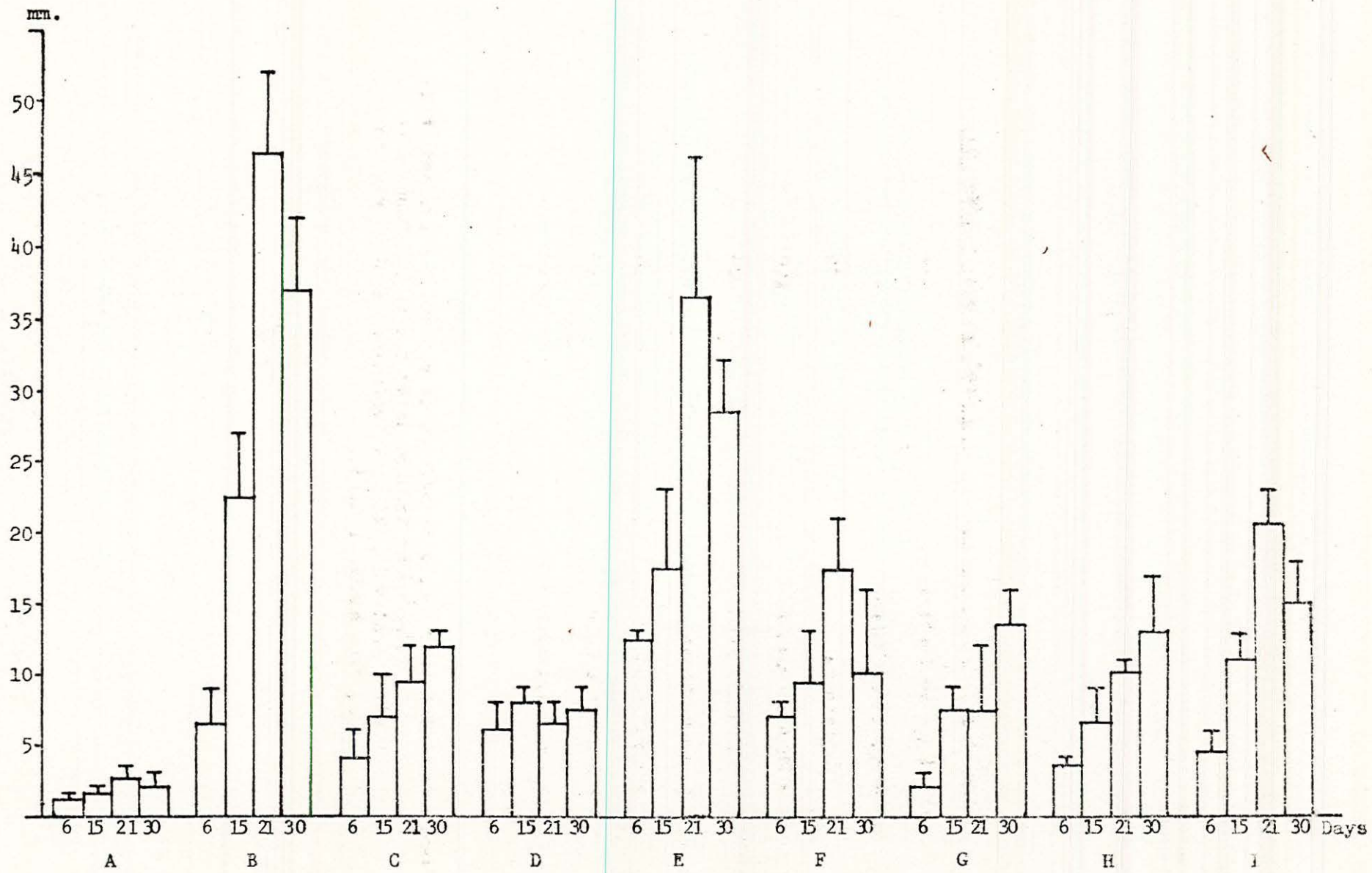
| Treatment Group | Differential White Blood Cell Counts (Lymphocytes, Neutrophils) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 6 | | Day 15 | | Day 21 | | Day 30 | |
| | Lymphs. % | Neuts. % | Lymphs. % | Neuts. % | Lymphs. % | Neuts. % | Lymphs. % | Neuts. % |
| Negative Control | 83 | 12 | 78 | 17 | 81 | 15 | 81 | 17 |
| Positive Control | 51 | 43 | 56 | 43 | 53 | 37 | 63 | 32 |
| Cryogenine | 68 | 28 | 67 | 27 | 66 | 29 | 71 | 25 |
| Phenylbutazone | 65 | 30 | 63 | 35 | 68 | 28 | 74 | 21 |
| Mefenamic Acid | 57 | 39 | 52 | 42 | 61 | 31 | 59 | 37 |
| Indomethacin | 51 | 47 | 57 | 37 | 65 | 29 | 63 | 33 |
| Paramethasone | 46 | 48 | 49 | 47 | 49 | 44 | 61 | 33 |
| Hydrocortisone | 42 | 53 | 51 | 45 | 57 | 39 | 73 | 22 |
| 6-mercaptopurine | 58 | 37 | 69 | 21 | 69 | 27 | 69 | 25 |

white blood cell responses to adjuvant-induced arthritis. It was evident that the adjuvant disease resulted in a biphasic increase in total white cell count which was associated with a relative lymphopenia and neutrophilia in positive control group animals. The first cell count increase was associated with the development of primary swelling in the injected foot, while the later increase (Day "21") was associated with the development of secondary lesions. Treatment with cryogenine and the other nonsteroidal antiinflammatory agents appeared to offer only a moderate degree of protection relative to the level of leucocytosis observed in positive control animals during the 30 day observation period.

The comparative effects of drug treatments on levels of sedimentation rates in animals injected with Mycobacterium adjuvant are illustrated in Figure 28. An increase in sedimentation rates was noted in positive control group animals within 6 days following adjuvant injection and was associated with the primary inflammatory response. A very significant (observed P= 0.01) elevation in sedimentation rates was observed on Day "15" during the acute phase of the disease and on Day "21" when secondary lesions were fully manifested. These values continued to remain elevated for the remainder of the 30 day observation period. In adjuvant-injected rats, cryogenine, phenylbutazone, hydrocortisone and paramethasone were found to be the most effective of the agents tested in suppressing the

| Treatment Group | Oral Dosage, mg./Kg./Day |
|---|---|
| A = Negative Control | --- |
| B = Positive control | --- |
| C = Cryogenine | 100 |
| D = Phenylbutazone | 100 |
| E = Mefenamic Acid | 25 |
| F = Indomethacin | 1.0 |
| G = Paramethasone | 0.5 |
| H = Hydrocortisone | 10 |
| I = 6-mercaptopurine | 2.0 |

Figure 28.-- Effects of drug treatments on erythrocyte sedimentation rate in adjuvant-induced arthritic rats. Drugs were given orally for 21 days beginning one day prior to adjuvant administration.


mm.
50
45
40
35
30
25
20
15
10
5
6 15 21 30
A
B
C
D
E
F
G
H
I
Days

increase in sedimentation rate, since values tended to approach normal control levels. Mefenamic acid at a dose level of 25 mg/Kg afforded little effect on sedimentation rates in comparison to positive control group animals receiving no drug treatment. The cytotoxic agent, 6-mercaptopurine, decreased sedimentation rate, but to a lesser extent than the nonsteroidal antiinflammatory compounds.

2. Ultraviolet-Induced Erythema

The efficacy of cryogenine, mefenamic acid, phenylbutazone and indomethacin in reducing or delaying the erythemic response to ultraviolet irradiation in the guinea pig is summarized in Table XII. The ED50 values, interpolated from log-dose response curves, represent the dose of each drug which was determined to inhibit the erythemic response by 50 percent in comparison to control group animals. At the dose levels employed, 6-mercaptopurine was inactive. As noted by other investigators (37,70) the steroidal compounds, paramethasone and hydrocortisone were essentially devoid of activity in this experimental model of inflammation.

3. Acute Cardiovascular Interactions

Intravenous doses of cryogenine acetate were essentially without effect on mean resting blood pressure in the rat. Cryogenine in doses of 0.04-8.0 mg/Kg produced only a slight and very transient decrease in blood pressure immediately following injection. Depressor

Table XII.-- Comparative effects of cryogenine on ultraviolet-induced erythema in the guinea pig

| Treatment | Oral dose mg./Kg. | Group inflammatory scores 1 hr | 2 hr | 3 hr | 3 hr integrated score | ED50 mg./Kg. |
|---|---|---|---|---|---|---|
| Cryogenine | 0 | 9.3 | 13.0 | 14.3 | 29.5 | 38 |
| | 16 | 6.3 | 7.3 | 11.7 | 19.5 | |
| | 32 | 4.3 | 6.7 | 11.0 | 16.5 | |
| | 64 | 2.7 | 4.7 | 8.7 | 11.8 | |
| Phenylbutazone | 0 | 9.0 | 13.3 | 14.3 | 29.5 | 12.5 |
| | 8 | 5.0 | 7.3 | 10.7 | 17.7 | |
| | 16 | 2.3 | 7.0 | 9.3 | 14.0 | |
| | 32 | 1.3 | 3.3 | 5.3 | 7.3 | |
| Mefenamic Acid | 0 | 9.3 | 13.0 | 14.7 | 29.7 | 23.5 |
| | 16 | 5.7 | 6.3 | 11.0 | 17.5 | |
| | 32 | 4.0 | 6.3 | 8.7 | 14.7 | |
| | 64 | 0.7 | 2.0 | 4.3 | 4.9 | |
| Indomethacin | 0 | 10.7 | 14.0 | 14.3 | 31.9 | 3.1 |
| | 2 | 6.3 | 7.3 | 11.7 | 19.5 | |
| | 4 | 4.3 | 5.0 | 9.3 | 14.0 | |
| | 8 | 2.0 | 4.3 | 7.0 | 9.8 | |
| Paramethasone | 0 | 9.3 | 12.7 | 14.3 | 29.2 | -- |
| | 16 | 9.0 | 13.0 | 14.0 | 29.0 | |
| | 32 | 8.7 | 13.0 | 13.3 | 28.4 | |
| | 64 | 9.0 | 12.0 | 14.0 | 28.0 | |
| Hydrocortisone | 0 | 10.3 | 12.7 | 13.7 | 29.9 | -- |
| | 16 | 11.0 | 14.0 | 14.0 | 32.0 | |
| | 32 | 9.7 | 13.0 | 13.7 | 29.6 | |
| | 64 | 8.7 | 13.3 | 13.3 | 28.7 | |
| 6-mercaptopurine | 0 | 9.0 | 12.3 | 13.7 | 28.2 | -- |
| | 16 | 9.0 | 12.7 | 14.0 | 28.7 | |
| | 32 | 8.7 | 11.7 | 12.3 | 26.6 | |
| | 64 | 9.0 | 12.0 | 12.3 | 27.2 | |

responses to bradykinin, histamine and acetylcholine challenges were only slightly altered from control levels following cryogenine treatment while pressor responses to epinephrine challenges were only slightly reduced (Figure 29.). However, cryogenine at doses of 4.0-8.0 mg/Kg suppressed the depressor response to test challenges of serotonin approximately 20 to 25 percent from control levels.

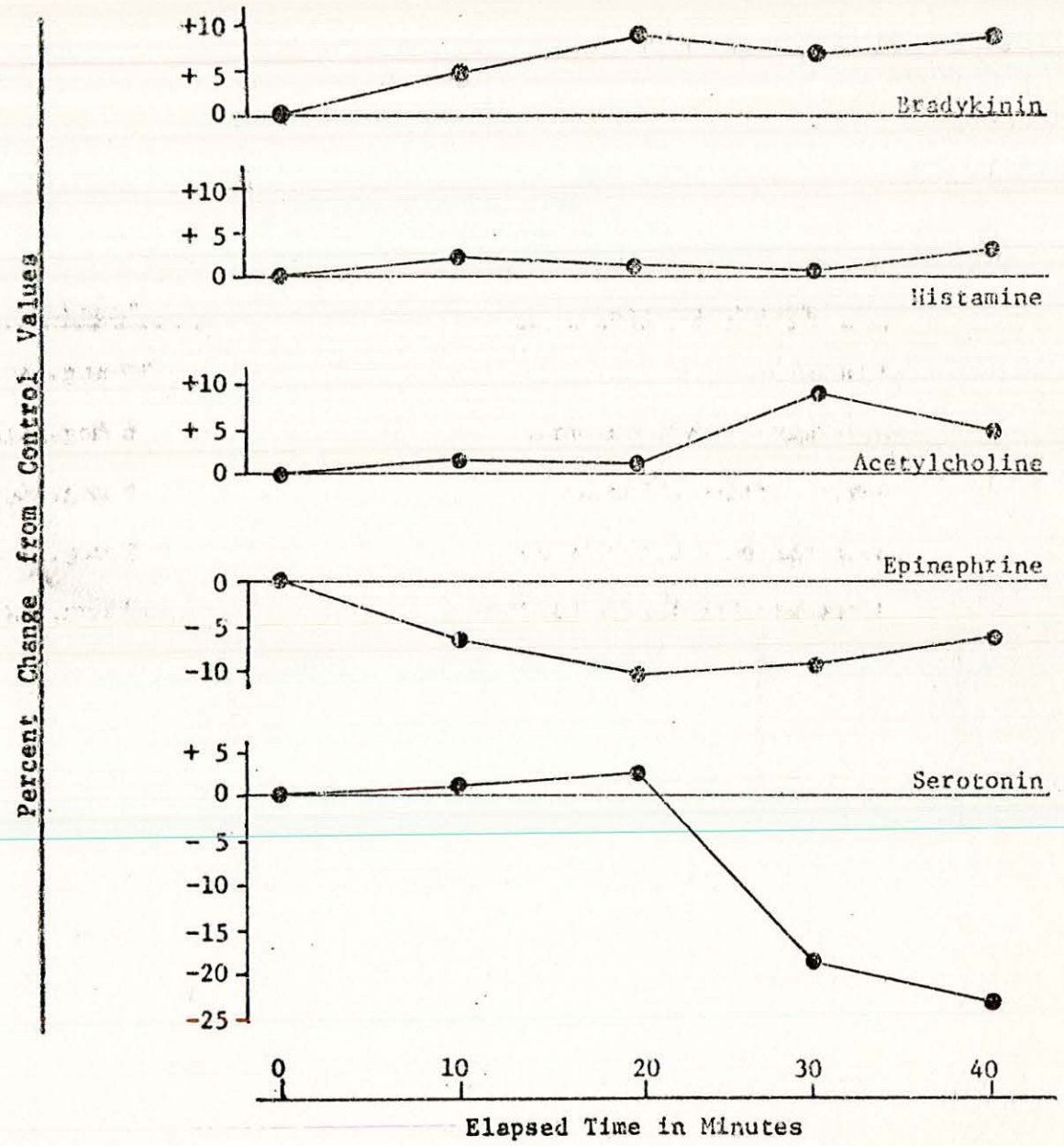
4. Drug-Induced Bronchoconstriction

In guinea pigs prepared for recording resistance of the lungs to inflation, control reference challenges of bradykinin were administered intravenously in doses ranging from 0.5 to 1.0 mcg/Kg. In all animals these doses of bradykinin increased resistance to inflation to a marked degree. Reference to Figure 30. indicates that the response to bradykinin differed from those to histamine and serotonin in its slower onset and longer duration at or near peak levels. The bronchoconstrictor action of bradykinin was found to be essentially unaffected by intravenous doses of cryogenine (5-10 mg/Kg) administered 5 to 20 minutes prior to the bradykinin challenge doses when compared to control reference responses.

Responses to histamine (2.0 mcg/Kg) obtained in the same manner, were often variable following cryogenine pretreatment (Figure 31.). Generally, however, little change from control responses was noted.

| Test Challenges | Dosage |
|---|---|
| Bradykinin | 10 mcg./Kg. |
| Histamine Dihydrochloride | 6 mcg./Kg. |
| Acetylcholine Chloride | 4 mcg./Kg. |
| Epinephrine Hydrochloride | 2 mcg./Kg. |
| Serotonin Creatinine Sulfate | 2 mcg./Kg. |

Figure 29.-- Acute cardiovascular interactions of cryogenine in the anesthetized rat. (mean response / 2 determinations)




| | | | | | |
|---|---|---|---|---|---|
| Cryogenine Dosage | 0 | 0.04 | 0.4 | 4.0 | 8.0 mg./Kg. |
| Cumulative Dosage | 0 | 0.04 | 0.44 | 4.44 | 12.44 |

Top Row:

● = Bradykinin 0.5 mcg./Kg.

▲ = Cryogenine 5.0 mg./Kg.

Middle Row:

● = Bradykinin 0.5 mcg./Kg.

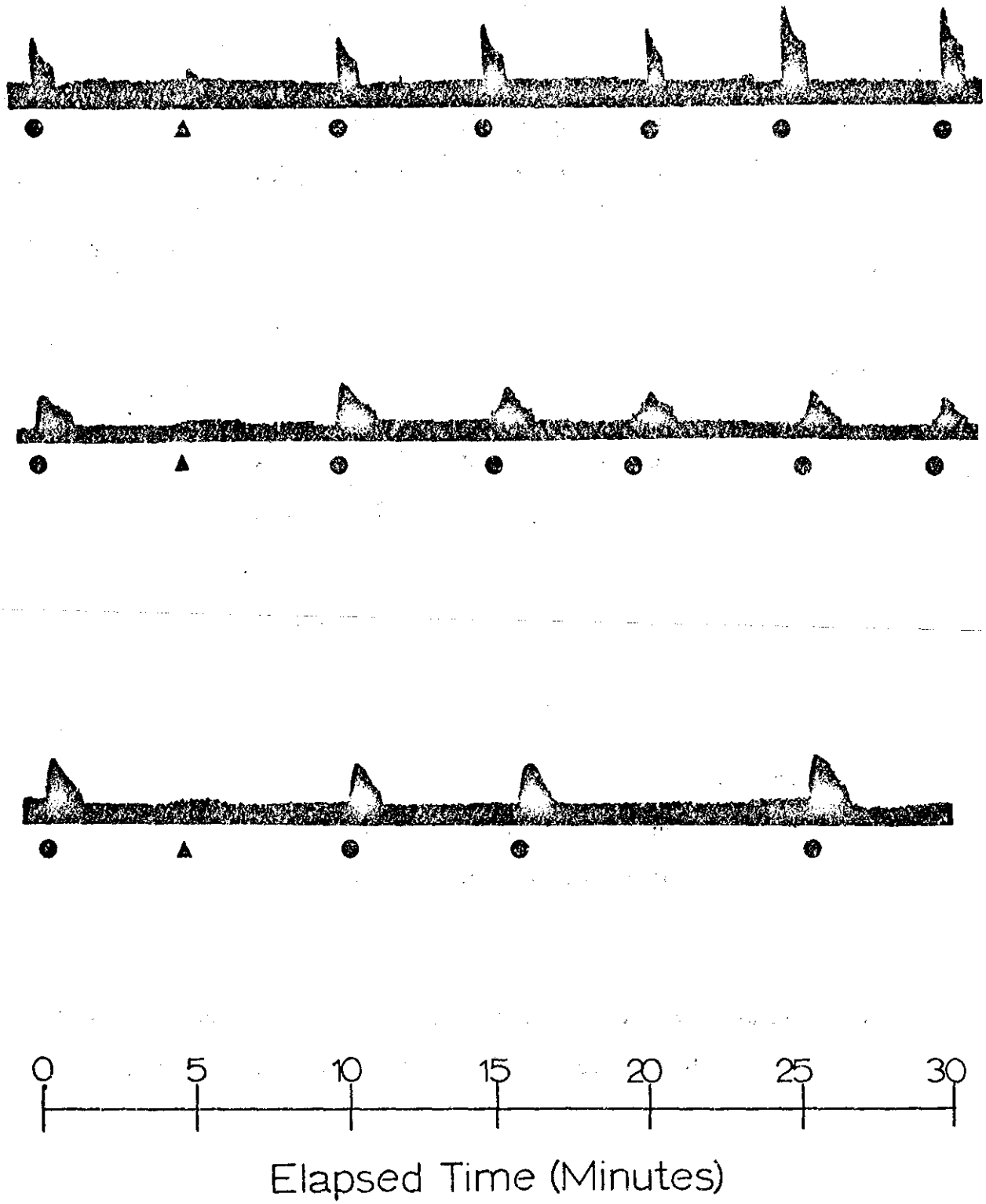
▲ = Cryogenine 10.0 mg./Kg.

Bottom Row:

● = Bradykinin 1.0 mcg./Kg.

▲ = Cryogenine 10.0 mg./Kg.

Figure 30.— Effect of cryogenine on bradykinin-induced bronchoconstriction in the guinea pig


0
5
10
15
20
25
30
Elapsed Time (Minutes)

Top Row:

● = Histamine 2.0 mcg./Kg.

▲ = Cryogenine 5.0 mg./Kg.

Middle Row:

● = Histamine 2.0 mcg./Kg.

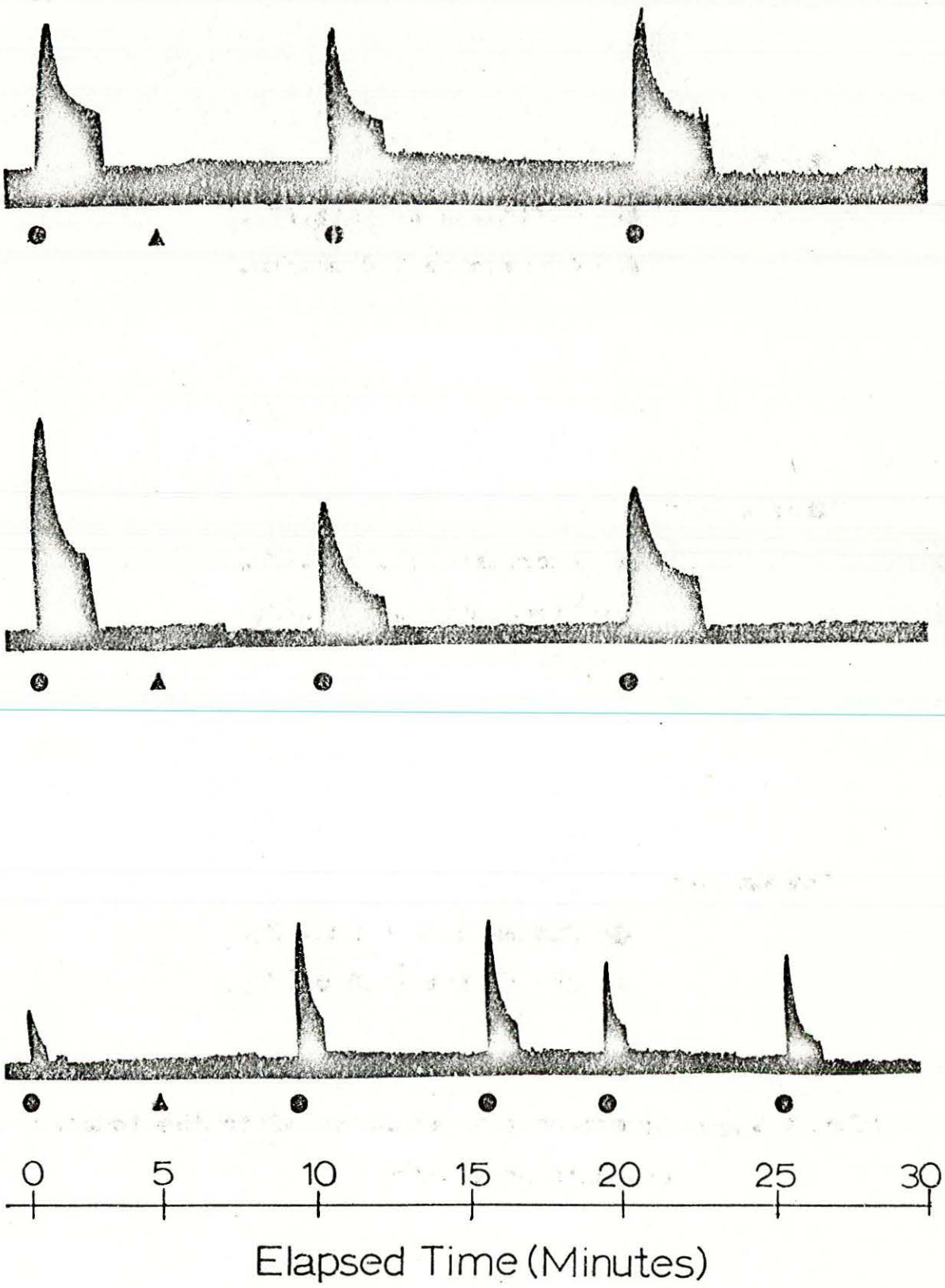
▲ = Cryogenine 5.0 mg./Kg.

Bottom Row:

● = Histamine 2.0 mcg./Kg.

▲ = Cryogenine 10.0 mg./Kg.

Figure 31.— Effect of cryogenine on histamine-induced bronchoconstriction


0
5
10
15
20
25
30
Elapsed Time (Minutes)

Cryogenine, injected 5 to 25 minutes before challenge doses of serotonin (1.25 mcg/Kg) consistently enhanced the bronchoconstrictor effect of the challenge drug to levels from two to three times those of control reference responses (Figure 32.).

5. Ganglionic Interactions

Cryogenine was administered intraarterially during continuous preganglionic stimulation at frequencies ranging from 0.1-1.0 cps. Following injection of the drug, ganglionic transmission was monitored continuously for a period of 2 hours. Cryogenine (50-750 mcg) failed to produce any detectable changes in either spike amplitude or afterpotential contour evoked by supramaximal or submaximal preganglionic stimulation. The submaximal stimulation was determined by selecting that voltage which would produce a ganglionic spike one-half the amplitude of that obtained with supramaximal preganglionic stimulation. It was determined that cryogenine (50-750 mcg) did not evoke ganglionic firing. Moreover, no changes in the resting demarcation potential were observed as the result of cryogenine administration. Similarly, there was no evidence of asynchronous postganglionic discharge evoked by cryogenine (750 mcg) in ganglia conditioned by repetitive supramaximal preganglionic stimulation (30 cps for 30 sec.) or in ganglia pretreated with isoproterenol (2 mcg).

Top Row:

○ = Serotonin 1.25 mcg./Kg.

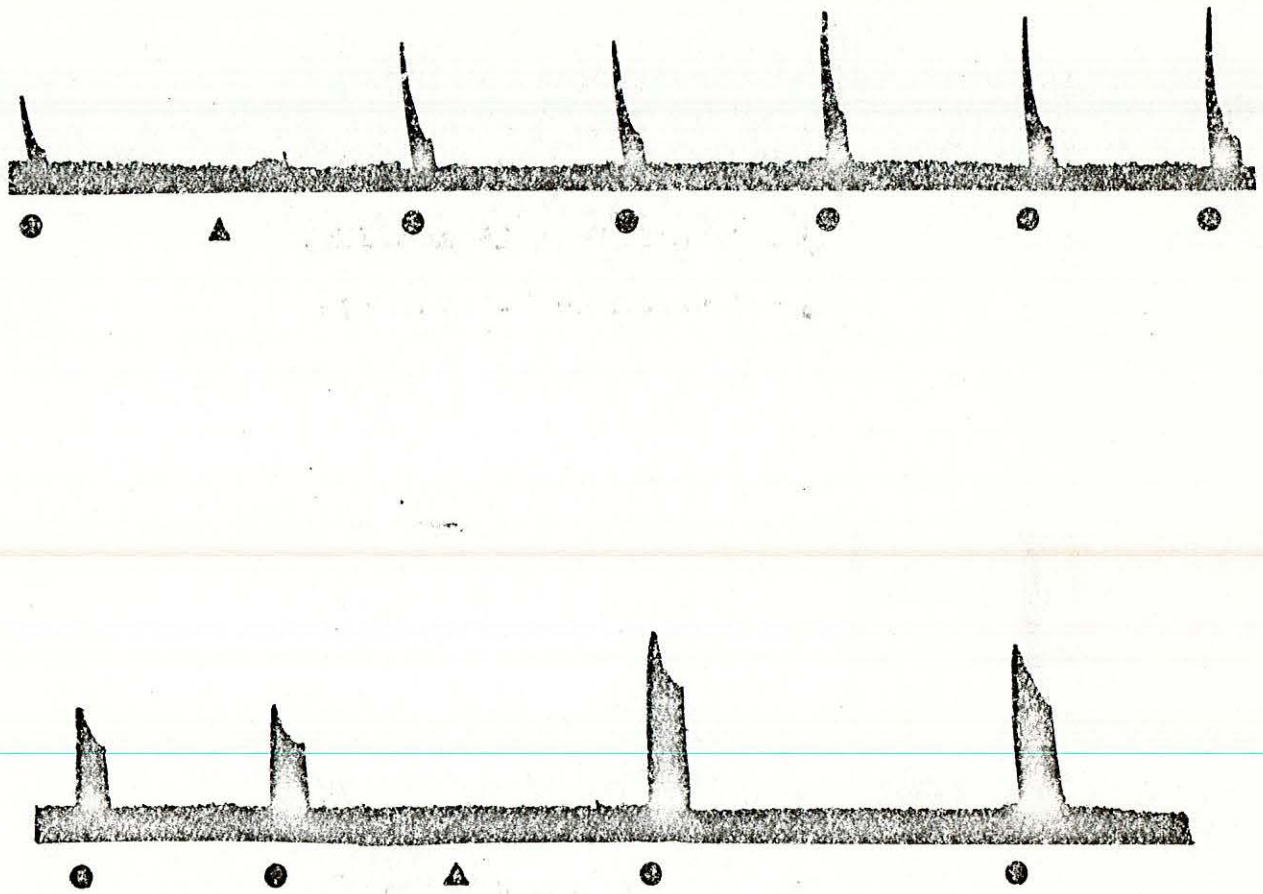
▲ = Cryogenine 5.0 mg./Kg.

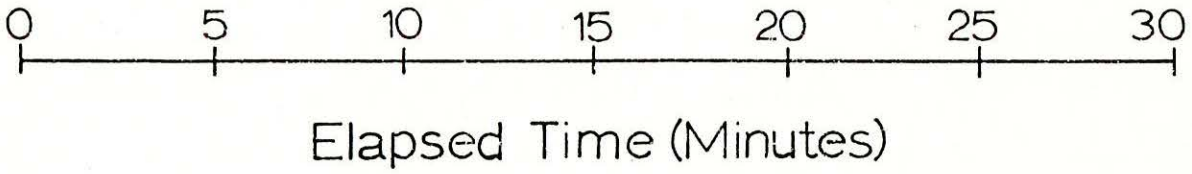
Bottom Row:

○ = Serotonin 1.25 mcg./Kg.

▲ = Cryogenine 10.0 mg./Kg.

Figure 32.-- Effect of Cryogenine on serotonin-induced bronchoconstriction in the guinea pig




0
5
10
15
20
25
30
Elapsed Time (Minutes)

Ganglionic discharges evoked by acetylcholine (10 mcg), DMPP (5 mcg), 5-HT (10 mcg) or KCl (500 mcg) were unaffected by cryogenine (750 mcg) administered 5 or 60 sec. earlier.

The postganglionic firing evoked by the intraarterial administration of TMA (5-10 mcg) was not modified in ganglia pretreated with cryogenine 15 sec. earlier. Furthermore, the biphasic ganglionic demarcation potential produced by TMA (10 mcg) and DMPP (5 mcg) was unaltered by the previous administration of cryogenine (750 mcg). The postganglionic firing produced in response to physostigmine (200 mcg), oxotremorine (75 mcg), methacholine and McN-A-343 (25 mcg) was unaffected by the administration of cryogenine (750 mcg) prior to or during the evoked asynchronous discharges. Moreover, there were no apparent alterations in the enhanced level of firing evoked by these agents in ganglia pretreated with isoproterenol (2 mcg) or in ganglia conditioned previously by repetitive supramaximal preganglionic stimulation (30 cps for 30 sec.). In addition, cryogenine (750 mcg) failed to prevent the complete abolition of the "muscarinic" firing that occurred almost immediately following the administration of atropine (2 mcg).

In ganglia pretreated with cryogenine immediately prior to or 30 sec. before injection of DMPP (5 mcg), TMA (10 mcg), levarterenol (0.5 mcg), epinephrine (0.5 mcg) or methacholine (50 mcg), no observable antagonism or

enhancement of the ganglionic blockade produced by these compounds was noted.

## DISCUSSION

A variety of biologic techniques and experimental approaches have been employed to discover potential antiinflammatory drugs for the treatment of human connective tissue diseases. To this end, there have been numerous attempts to produce an arthritis-like state in laboratory animals during the last seventy years. In 1960, Gardner (38) reviewed and classified these attempts to artificially induce arthritic and inflammatory states by the various means employed: (a) infective agents, (b) chemical agents, (c) alterations of endocrine mechanisms, (d) immunological and (e) physical methods.

In 1937, Freund et al. (39) published their method of sensitizing guinea pigs and rabbits by injection of killed tubercle bacteria. In later work, when the organisms were suspended in mineral oil and injected, the sensitivity and antibody titres produced were found to be markedly increased. Subsequently, this method was observed

to produce an arthritic-like condition in rats. The mineral oil's adjuvant function as the carrier of the Mycobacterium is unknown. However, it has been suggested that it may aid in dissemination from the injection site, prevent destruction and protect the dead organisms from phagocytosis (56). Since that time, numerous investigators have shown that a single injection of Freund's adjuvant (containing any one of a number of species of Mycobacterium) will produce a chronic, local and migratory inflammatory response in the rat (27,35,41-44). The lesions which subsequently develop involve not only joints and surrounding tissue (synovitis, periarthritis, spondylitis), but also skin (nodules of ears, feet, tail) and eyes (conjunctivitis, keratitis) (45). The disease has many similarities to rheumatoid arthritis and other connective tissue disorders which occur in man. Thus, the polyarthritic syndrome in rats has become one of the most important models of inflammation for the study of potentially useful steroidal and nonsteroidal antiinflammatory agents.

The etiology of this arthritic disease in rats is still unknown (46,47). However, a body of indirect evidence is accumulating which indicates that an immunological reaction to unknown constituents of the tubercle bacilli may be involved.

i. The experimental disease can be transferred to normal rats with viable lymph node cells

obtained from sensitized donor rats of the same inbred strain (46).

ii. Induction of tolerance to mycobacterial antigen in the neonatal period (before 5 weeks old) can inhibit subsequent production of the disease (48,49).

iii. There is a characteristic 10-15 day latent period to the onset of polyarthritis (41).

iv. Anti-rat-lymphocyte serum inhibits the appearance of arthritis (50,51).

v. Whole body irradiation or the administration of corticosteroids will suppress the arthritic condition (41).

The possibility that an infectious agent is responsible for the pathogenesis of this disease is generally considered to be unlikely since the disease can be induced in gnotobiotic rats and its course is not influenced by massive pretreatment with penicillin, streptomycin or tetracycline (41). Moreoever, at the sites where secondary lesions arise, tubercle bacteria or their constituents cannot be demonstrated. Mycoplasmatacea (PPLO) and the activation of a latent virus by an immune reaction have been inferred by some investigators to be responsible for initiating lesion formation, but as yet, this has not been proven (52,53).

While the responsible antigen has not been identified,

Jones and Ward (54) have shown that $^{14}C$ labelled mycobacteria suspended in mineral oil are rapidly disseminated after intradermal injection and can be found in lymph nodes, spleen and periarticular tissues within a few hours after injection. It is also possible that antigens other than those incorporated into the adjuvant could be evoked. Tissue antigens at the depot site have, however, been excluded by investigators who have shown that removal of the injection site (the tail) within several hours after injection with adjuvant does not influence the development of polyarthritis (55).

Mycobacterium-induced arthritis is a disease peculiar to rats. Glenn and Gray (56) were unsuccessful in inducing the disease in hamsters, mice, gerbils, guinea pigs, or rabbits. Similar attempts to produce the disease in boars, male monkeys, dogs and roosters were likewise without success. The arthritis is permanently deforming because of the extensive connective tissue involvement.

The initial reaction of edema and soft tissue thickening at the injection site is probably due to the irritant effect of adjuvant, whereas the late onset disseminated polyarthritis and the late swelling in the injected foot are presumably immunologic events. Different rat strains vary somewhat in response to the induced disease; however, neither sex differences nor thymectomy, splenectomy, bile duct ligation, castration or pregnancy alter the course or incidence of the disease. Adrenalectomy

and hypophysectomy result in death soon after adjuvant administration (56).

Many methods employed in the pharmacologic screening of antiinflammatory agents utilize the acute inflammation produced by the injection of materials such as dextran, formalin, yeast, silver nitrate, or various suspected mediators of inflammation and other agents too numerous to mention here. Unlike the inflammatory syndrome associated with connective tissue disorders such as rheumatoid arthritis, the inflammatory response to the above injected agents is usually transitory and is relatively insensitive to clinically useful antiinflammatory drugs except at near toxic doses (57).

Other inflammatory lesions produced by sustained stimuli, such as the subcutaneous implantation of cotton pellets or the use of various granuloma pouch techniques are sensitive to antiinflammatory steroids but relatively insensitive to nonsteroidal agents (58).

Although there is some disagreement regarding the etiology and nature of adjuvant disease in rats, many of the compounds which have been proven useful in the treatment of arthritis in man have shown activity against one or more of the manifestations of the adjuvant disease in rats. These include both steroids and nonsteroids and various immunosuppressive agents (27,53,59,60). Adjuvant-induced arthritis in rats resembles rheumatoid arthritis in man more than any other model of inflammation in current

use.

Methods used to measure inflammatory manifestations in the arthritic rat are extremely important. Numerous subjective scoring systems have been devised. The magnitude of the swelling of paws has been determined by measuring the circumference of inflammed limbs or by determining the thickness of hind feet with calipers (27). The edematous response, however, is frequently not uniform and is often not localized in the areas being measured. Furthermore, these methods are also partially subjective when judging the uniformity of pressure to be exerted by the calipers on the soft tissue. Moreover, the possibility of aggravating the inflammed tissues exists, especially in chronic studies which require repeated measurements. In the present study, the plethysmographic determination of paw volume was used to evaluate the activity of antiinflammatory drugs. Employing this method, Winter and Nuss (59) were able to obtain accurate, objective and reproducible results related to log-dose administration of drugs. Furthermore, the high level or precision obtained with this system of measurement permits a statistical treatment of data not possible by the other means.

The results of the present study show cryogenine to be effective in significantly suppressing the development of adjuvant-induced arthritis in doses which appeared to be devoid of any toxic effects. Rats treated with

cryogenine during the course of the disease remained conspicuously healthy when compared to the positive-control animals which lost weight, developed coarse ruffled fur and generally appeared ill. In addition, more spontaneous activity and much less irritability was observed in rats treated with cryogenine. In the present experiments, untreated rats began to fight and vocalize and assume defensive "fighting" postures within a week after adjuvant injection (56). As the course of the disease progressed this type of activity became more evident. Such behavior was noticeably absent in rats treated daily with cryogenine, an observation tending to confirm the early reports of the mild tranquilizing properties inherent to the drug (13).

In this investigation, test drugs were administered in doses which had been previously found to be optimal using other antiinflammatory testing procedures in our laboratory. None of the agents tested markedly prevented the swelling of the injected paw immediately after adjuvant administration; however, an early arrest of swelling (but not reversal was achieved by cryogenine and most of the other therapeutic agents. Mefenamic acid was an interesting exception. In addition, secondary lesions in non-injected paws (as well as ear and tail) were markedly inhibited by all of the agents studied during treatment. Moreover, a relatively long lasting antiinflammatory effect was observed <u>after termination of treatment</u> with

cryogenine, paramethasone, 6-mercaptopurine and phenylbutazone, while animals treated with indomethacin and mefenamic acid showed some relapse when treatment ended. In addition, cryogenine produced a weight gain in animals in which the disease was allowed to develop for three weeks before treatment was initiated. Moreover, the swollen feet in these rats actually regressed within two days of treatment with the alkaloid.

Following injection of adjuvant into a rear paw there was swelling accompanied by ulceration and sometimes sloughing and hemorrhage at the injection site. These manifestation obviously added to the animals' general debility. Moreover, there was an abrupt decrease in body weight just prior to the appearance of secondary lesions. Measurements of body weight during and after drug treatment indicated that among the nonsteroidal antiinflammatory drugs which significantly suppressed the polyarthritic condition, only cryogenine was effective in permitting a partial restoration of the normal rate of body weight gain.

Recent reports by Newbold (61) and Piliero et al. (62) suggested that a biphasic increase in leucocytes occurs during the development of adjuvant-induced arthritis. The results of the present study confirmed that an initial rise in leucocytes did take place and was associated with the phase of primary swelling. A subsequent rise in leucocyte count was also confirmed and this rise is associated with the development of the secondary lesions.

Our results also revealed a persistence of the leucocytic response throughout and (probably) extending beyond the 30 day observation period. The beneficial effects of cryogenine were evidenced by the fact that little change from normal levels occurred during drug treatment. The therapeutic efficacy of cryogenine was also evident in the differential white blood cell counts. In control and drug-treated rats, the increases in total leucocyte counts were due primarily to increases in the number of neutrophils, which was correlated with a relative lymphopenia and neutrophilia. Cryogenine was again the most effective nonsteroidal agent in modifying the ratio of polymorphonuclear leukocytes to lymphocytes towards normal. In addition to an absolute leucocytosis, arthritic control animals exhibited the classical responses to stress -- including adrenal and splenic hypertrophy and thymic involution. The therapeutic efficacy of cryogenine in suppressing this apparent adrenocortical overactivity was manifested by adrenal and thymus weights in adjuvant-treated animals which were not markedly different from those of control animals without adjuvant injection. Cryogenine differed from paramethasone, hydrocortisone and 6-mercaptopurine in that it did not prevent splenic enlargement during drug treatment. Both paramethasone and hydrocortisone caused involution of the adrenals, no doubt due to their ACTH "blocking" action.

These studies also revealed an increase in the

erythrocyte sedimentation rate that paralleled the rises found in total leucocyte counts in adjuvant-injected animals. It is interesting to note that similar changes have also been observed in patients with rheumatoid arthritis (63, 64). Cryogenine and most of the therapeutic agents tested were significantly protective against this manifestation of the inflammatory response. While increased sedimentation rate is a nonspecific reaction, this metameter appears to be a rather convenient and valuable index for assessing the therapeutic efficacy of drugs in this model of inflammation because of the small amount of blood sample needed and the relatively minimal expense in both time and equipment.

In a recent study, Piliero and Colombo (65) investigated the action of antiinflammatory drugs on the lysozyme activity and turbidity of serum from rats with adjuvant disease. Increased lysozyme levels and decreases in serum turbidity (as determined by the stability of serum protein against heat denaturation) were found to have an excellent correlation with the severity of the various manifestations of the polyarthritic condition. Treatment with various therapeutically useful agents produced a reversal of effect for both indicators of inflammation. Weissman (66) has proposed that degradative enzymes released from lysosomes may denature the native constituents of cells of connective tissue. These denatured products may in turn induce an immune response like that seen in adjuvant-induced arthritis.

Studies of lysozyme activity of supernatants of homogenates of inflammed paws from arthritic rats revealed very significant activity relative to noninflammed paws, where there was virtually an absence of lysozyme (65). The mechanisms whereby antiinflammatory agents inhibit lysozyme activity are essentially unknown; however, it has been suggested that inflammation is decreased by substances that stabilize lysosomal enzymes, interfere with the formation of antigen-antibody complexes (which upon ingestion by leucocytes, could release lysosomal enzymes) or inhibit the migration of lysosome-containing white cells to areas of inflammation (67,68). Whether the decrease in heat-induced serum turbidity is directly related to elevated lysozyme activity is unresolved as yet; however, it is of interest to note that the reversals in serum lysozyme levels produced with antiinflammatory drugs correlate well with those seen in serum turbidity.

The present investigation confirmed the observations of Piliero and Colombo (65) in that doses of the steroidal and nonsteroidal drugs which effectively suppressed inflammatory manifestations of adjuvant-induced arthritis also produced significant reversal effects in serum turbidity towards normal levels. Cryogenine was shown to be essentially equipotent to phenylbutazone in this regard.

2. Ultraviolet-Induced Erythema

One of the more widely used techniques for anti-inflammatory screening is that of ultraviolet-induced

erythema in the guinea pig. This method was originally introduced by Wilhelmi (69). The vascular response of guinea pig skin to ultraviolet light has several components. An early histamine and/or serotonin-mediated phase of increased vascular permeability develops within 2 or 3 minutes, lasts for about 10 minutes and is followed after about 1 hour by the appearance of erythema (70). This increase in capillary permeability provides a rapid and sensitive means for detecting nonsteroidal antiinflammatory agents since there is a good empirical correlation between the ability of a drug to delay development of erythema and its antirheumatic action in the clinic (31). It should be noted, however, that a number of vasodilator and ganglioplegic drugs which are active in this test, have no clinical utility in human rheumatic disease (71). Moreover, corticosteroids are inactive in this screening procedure (31). The results of the present study confirm the anti-erythemic actions of phenylbutazone, mefenamic acid and indomethacin and also show that cryogenine possesses significant anti-erythemic efficacy. Phenylbutazone was determined to have an acute (oral) potency approximately 3 times that of cryogenine in this procedure.

3. Acute Cardiovascular Interactions

Previous reports have indicated that in vitro cryogenine inhibits smooth muscle contractions elicited by acetylcholine and suspected mediators of the inflammatory response (histamine, serotonin, bradykinin) in a non-competitive manner (13, 14). Early cardiovascular studies

by Robichaud et al. (12) conducted in the dog and cat demonstrated cryogenine to have little effect on resting blood pressure, however, a later study by Kaplan et al. (15) in one dog showed cryogenine to significantly lower resting blood pressure. Drugs affecting cardiovascular integrity are known to alter inflammatory responses in a nonspecific manner and thus cannot be regarded as true antiinflammatory agents. Garattini et al. (72) demonstrated that hypotensive drugs simulate significant anti-inflammatory activity especially in those tests utilizing rapidly developing edema formation.

The present study indicated that cryogenine essentially is devoid of any effect on resting blood pressure in the rat at the relatively high dose levels employed. Moreover, only equivocal variations in response to histamine, acetylcholine, epinephrine and bradykinin challenges were observed. The only significant cardiovascular finding was the progressive inhibition of the serotonin-induced depressor response. The pharmacological significance of this effect, however, is obscure at this time.

4. Drug-Induced Bronchoconstriction

One of the more interesting methods for evaluating the antiinflammatory action of drugs is to test them as specific inhibitors of the actions of the suspected humoral mediators of inflammation. Bradykinin has been implicated in the early stages of the acute inflammatory

process because it produces vasodilation, increases capillary permeability, causes edema and leucocyte infiltration and elicits pain (73). This implication is further supported by the observation that bradykinin-induced bronchoconstriction in the guinea pig is antagonized by a large number of clinically useful antiinflammatory agents (74). However, bradykinin antagonism by antiinflammatory agents cannot be demonstrated in many other in vivo preparations (rat blood pressure, edema and erythema in guinea pig or rabbit skin, rat uterus contractions). Because antiinflammatory agents inhibit only the effects of bradykinin on guinea pig bronchi but not on other organs; Collier (75) has proposed that there are two receptors for bradykinin. As noted previously, a close correlation for nonsteroidal inflammatory agents has been observed between potency in delaying guinea pig skin erythema after exposure to ultraviolet irradiation and activity against rheumatic diseases in man. Moreover, potency in delaying the erythemic reponse has been correlated with that of antagonizing bradykinin-induced brochoconstruction in the guinea pig (74). For these reasons, it was of interest to determine to what extent cryogenine would interfere with the bronchoconstrictor action of bradykinin. The results of this study demonstrated that cryogenine failed to antagonize the bradykinin-induced increase in resistance to inflation of guinea pig lungs in vivo; in this way cryogenine differs

from indomethacin, mefenamic acid and phenylbutazone (44).

5. Ganglionic Interactions

In one of the first papers published on the pharmacology of cryogenine, Robichaud et al. (12) reported that the intraperitoneal administration of cryogenine would produce mild hypothermia, decreased motor activity, mydriasis, skin blanching and pilomotor erection in unanesthetized rats. At relatively low non-ataxic dosage levels, Robichaud et al. (13) noted that cryogenine would selectively suppress conditioned avoidance responding, in both discrete and continuous, avoidance-escape situations. Cardiovascular studies in the anesthetized intact dog and cat demonstrated a significant blockade of the pressor response to exogenously administered epinephrine with doses of cryogenine having no apparent effect on resting blood pressure (12). However, kinetic experiments on various isolated tissue preparations demonstrated an apparent lack of specificity of cryogenine for the commonly employed cholinergic and adrenergic receptor systems (14,15,17). Robichaud also studied the effects of cryogenine in two cats using the classical superior cervical ganglion-nictitating membrane preparation. All drug injections were made via the femoral vein and drug was allowed to cumulate in the animal until death resulted. No ganglionic blockade was seen in one cat up to the time of death, while a 50 percent blockade was seen in the other animal. Therefore, it was of

importance to us to document unequivocally whether or not cryogenine does possess any ganglionic activity, since such activity could produce false-positive results in antiinflammatory testing (72). There are certain structural similarities between the lupine alkaloids (cytisine and sparteine) and some of the possible metabolic breakdown products of cryogenine. While the lupine alkaloids may possess a nicotine-like common denominator, several investigators have demonstrated distinct differences between them and cryogenine in regard to cardiovascular, antiinflammatory, and psychopharmacologic activity (13,15,76). This study supported these conclusions since cryogenine was essentially devoid of any ganglionic activity at the relatively high dose levels employed.

## CONCLUSIONS

Most antiinflammatory drugs are acidic compounds; therefore, the efficacy of cryogenine, an alkaloidal base, is of more than routine interest. Although cryogenine is structurally unrelated to any other known class of antiinflammatory, antipyretic, or analgesic agents it is effective in certain experimental inflammatory conditions which respond to the antiinflammatory drugs presently in clinical use. These models of inflammation include: adjuvant-induced arthritis, ultraviolet-induced erythema (indicated in our studies) as well as carageenin-induced edema, peptone-induced fever, and histamine-induced intradermal wheals (noted in previous studies (15)).

Cryogenine is active in inhibiting various suspected mediators of inflammation in smooth muscle preparations (histamine, acetylcholine, serotonin, bradykinin). Moreover, cryogenine possesses some selective central nervous system depressant activity. Its nonspecificity of action

against the common neuro- and tissue- hormones suggest that its antiinflammatory effects are not related primarily to the inhibition of acute phase reactants.

Involvement of the pituitary-adrenal axis in the activity of cryogenine seems improbable, since highly effective, but nontoxic doses of cryogenine do not affect body weight as the adrenocortical steroids do. Adrenal and thymus weights, which are sensitive indicators of adrenal stimulation, essentially remain unchanged by effective antiinflammatory doses of cryogenine. In addition, unlike steroidal antiinflammatory drugs, cryogenine does not reduce adrenal size or produce thymolysis on repeated administration.

Cryogenine has been shown to possess a number of properties in common with clinically useful antirheumatic drugs such as phenylbutazone, indomethacin and aspirin. It is effective in suppressing various inflammatory manifestations of adjuvant-induced polyarthritis in the rat and inhibits ultraviolet-induced erythema in the guinea pig. Experiments in which cardiovascular responses in rats were determined after cryogenine administration indicated that the inhibitory effects on edema formation are unlikely to be due to hypotension. The present study has also shown cryogenine to be essentially devoid of ganglionic activity. While the antiinflammatory efficacy of cryogenine does not appear to be related to peripheral neurotropic activity, its action may be mediated, at least

in part, through the central nervous system.

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